PrP gene expression regulation in sheep

Identification of candidate transcription factors that exhibit differential binding to polymorphic variants of the ovine PrP gene promoter

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TABLE OF CONTENTS

DECLARATION

I declare that the work carried out in this thesis is my own original work, except where otherwise stated. No part of this thesis has been, or will be submitted for any other degree or professional qualification.

ABSTRACT

Amino acid variants of PrP^c particularly at codons 136, 154 & 171 have been linked to scrapie susceptibility, but do not explain all variation in disease phenotype. This study has therefore addressed the hypothesis that unexpected disease occurrence may also be linked to different levels of PrP gene expression. In order to investigate the role of the ovine PrP gene 3'UTR in the regulation of gene expression, a series of ovine PrP mini-gene constructs were produced, which differed only in their availability of previously identified polyadenylation signals. Following the transient transfection of ovine and murine cell lines with these constructs it became clear that they were being incorrectly spliced, despite the fact that sequencing confirmed the presence of all of the elements required for correct splicing to occur.

Gene transcription is also regulated by the binding of sequence specific transcription factors to the promoter region and the role of the PrP gene promoter in the regulation of gene expression was investigated in the second half of this thesis. Sequence analysis using online and offline database resources revealed the presence of a number of transcription elements within the ovine PrP gene promoter. It was therefore decided to test the hypothesis that the binding of transcription factors to the ovine PrP promoter could influence the expression of the PrP gene. Using gel shift assays specific binding was observed to selected sequence elements and differential binding was demonstrated to a polymorphic variant of at least one of these motifs.

In addition, binding to four motifs conserved in the mammalian PrP promoters was also analysed using a combination of gel shift assay and DNase I footprinting. Two of the four motifs showed specific binding and polymorphic variants of these motifs exhibited differential binding. The factors bound to these sites were identified by gel super-shift assays using antibodies specific for the candidate proteins. A specific interaction was shown between the transcription factors bound to two of these motifs. This study has demonstrated the functionality of a number of transcription elements within the ovine PrP promoter and has provided further insights into the role of the promoter region in the regulation of PrP gene expression.

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ABBREVIATIONS

A	Alanine (amino acid) or adenine (nucleic acid), depending on context
Ala	Alanine
AP-1	Activator protein-1 or ovine AP-1 oligonucleotide, depending on context
AP-2	Activator protein-2
APP	Amyloid precursor protein
APS	Ammonium persulphate
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BABs	Animals born after the 1988 ban on the feeding of ruminant material to other ruminants
BAP	Bacterial alkaline phosphatase
BARBs	Animals born after the 1996 ban (termed the real ban) on the feeding of meat and bone meal to any food animal species
β-gal	Beta-galactosidase
bp	Base pair
BoPrP	Bovine PrP
BSE	Bovine spongiform encephalopathy
bZIP	Basic leucine zipper protein
C	Cytosine (nucleic acid) or cysteine (amino acid), depending on context
°C	Degrees Celcius
cAMP	Adenosine-3'-5'-cyclic monophosphate
CAT	Chloramphenicol acetyltransferase
CAT box	CCAAAT box DNA sequence
C/BP	cAMP binding protein or C/BP oligonucleotide, depending on context
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/ enhancer binding protein
CER I	Cytoplasmic extraction reagent-1
CER-II	Cytoplasmic extraction reagent-2
CFTR	Cystic fibrosis transmembrane conductance receptor
CH1641	Cheviot scrapie brain homogenate
ChIP	Chromatin immunoprecipitation
CHO	Chinese hamster ovary
CIAP	Calf intestinal alkaline phosphatase
CJD	Creutzfeldt-Jakob disease
cm	Centimetre
CNS	Central nervous system
CO_2	Carbon dioxide

cpm	Counts per minute
CPSF	Cleavage and polyadenylation specificity factor
CREB	cAMP response element binding protein
CREM-τ	cAMP-responsive element modulator-tau
CsCl	Caesium chloride
CSF	Cerebrospinal fluid
^{Ctm}PrP	Carboxy-terminally attached PrP
C-terminal	Carboxy-terminal
CWD	Chronic wasting disease
Cys	Cysteine
D	Aspartic acid
Da	Daltons
dAP-2s	Downstream AP-2 motif (Suffolk)
dAP-2c	Downstream AP-2 motif (Cheviot)
dNTP	2'-deoxynucleotide-5'-triphosphate
ddNTP	2'-3'-dideoxynucleoside-5'-triphosphate
DEFRA	Department for the environment food and rural affairs
dH_2O	Distilled H_2O
DIM	Detergent insoluble microdomain
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DNaseI	Deoxyribonuclease I
DPE	Downstream promoter element
Dpl	*Prnd* gene product designated doppel
dsDNA	Double stranded DNA
DTT	Dithiothreitol
E	Glutamic acid
E4BP4	bZIP repressor protein
EDTA	Ethylene diaminetetraacetic acid
EGF	Epidermal growth factor
EGR-1	Early gene transcription factor-1 or EGR-1 oligonucleotide, depending on context
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
F	Phenylalanine
fCJD	Familial Creutzfeldt-Jakob disease
FCS	Fetal calf serum
FDC	Follicular dendritic cells
FFI	Fatal familial insomnia
FLAG™	Tagging epitope FLAG™
3X FLAG™	Three adjacent copies of the tagging epitope FLAG™
FSE	Feline spongiform encephalopathy
FTIR	Fourier transform infrared spectroscopy
g	Gravitational force or grams, depending on context
G	Glycine (amino acid) or guanine (nucleic acid), depending on context
GATA-1	GATA-1 oligonucleotide
Gln	Glutamine

Glu	Glutamate
Gly	Glycine
GPI	Glycsylphosphatidylinositol
GSS	Gerstmann-Straussler-Scheinker syndrome
GTF	General transcription factor
H	Histidine
HAT	Histone acetyltransferase
HC	Heavy chain of precipitating antibody
hGH	Human growth hormone
His	Histidine
HIV-1	Human immunodeficiency virus
hM1/M2	Human motif 1 wild type/ motif 2 non-ruminant double oligonucleotide
hovM1/M2	Humanised ovine motif 1 variant/ motif 2 ruminant double oligonucleotide
hr	Hour
HSE	Heat shock element
HSE-1	Heat shock element-1 oligonucleotide
HSE-2	Heat shock element-2 oligonucleotide
HSF	Heat shock factor
HuPrP	Human PrP
I	Isoleucine
IAH	Institute for Animal Health
IAP	Intracisternal A-particle
IFN-α	Interferon-alpha
IFN-β	Interferon-beta
IFN-γ	Interferon-gamma
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
Ile	Isoleucine
Inr	Initiator element
IP	Immunoprecipitation
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IS120Cer	Cerebella derived cell culture from Icelandic sheep – genotype ARR/ARR
IS120Kid	Kidney derived cell culture from Icelandic sheep – genotype ARR/ARR
IS120Liv	Liver derived cell culture from Icelandic sheep - genotype ARR/ARR
IS120Med	Medulla derived cell culture from Icelandic sheep - genotype ARR/ARR
K	Lysine
kb	kilobase
kDa	kilo Dalton
kg	Kilogram
l	Leucine or litre, depending on context

LB broth	Luria-Bertani broth
LC	Light chain of precipitating antibody
Leu	Leucine
LRP	Laminin receptor protein
LyF-1	Lymphocyte-specific DNA-binding protein
Lys	Lysine
SCM	Standard complete medium
M	Methionine or molar, depending on context
M1C	Motif 1 variant oligonucleotide
M1T	Motif 1 wild type oligonucleotide
M1C/M2C	Motif 1 variant/ motif 2 ruminant double oligonucleotide
M2C	Motif 2 ruminant oligonucleotide
M2T	Motif 2 non-ruminant oligonucleotide
M3	Motif 3 oligonucleotide
M4i	Motif 4 (i) oligonucleotide
M4ii	Motif 4 (ii) oligonucleotide
M4iii	Motif 4 (iii) oligonucleotide
mA	Milli amp
MBM	Meat and bone meal
Met	Methionine
mg	Milligram
ml	Millilitre
mM	Millimolar
mm	Millimetre
M-per	Mammalian protein extraction reagent
ME7	A mouse passaged scrapie strain
MEM	Modified Eagle's medium
Mem-per	Mammalian membrane protein extraction reagent
mg	Microgram
MoPrP	Mouse PrP
MPP	Mg^{2+} dependent protein phosphatase
mRNA	Messenger ribose nucleic acid
n	Any nucleic acid
N	Adenosine, cytosine, guanine or thymidine (nucleic acid) or asparagine (amino acid), depending on context
N2a	Neuroblastoma cell line
NC-1	Non-competitor-1 oligonucleotide
NC-2	Non-competitor-2 oligonucleotide
NE	Nuclear extract
NE-PER	Nuclear and cytoplasmic extraction reagent
NER	Nuclear extraction reagent
NF-AT	Nuclear factor of activated T cells
NF-IL3	Nuclear factor of interleukin-3
NF-IL6	Nuclear factor of interleukin-6
NF-κB	Nuclear factor kappa B
ng	Nanogram
NGF	Nerve growth factor
N-Glycosylation	Asparagine (N) linked glycosylation

nm	Nanometre
nmol	Nanomoles
NMR	Nuclear magnetic resonance
NPU	Neuropathogenesis Unit, Institute for Animal Health
NSE	Neurone specific enolase
NSP	National scrapie plan
N-terminal	Amino-terminal
^{Ntm}PrP	Amino-terminally attached PrP
NCM	Nerve growth factor (NGF) complete medium
nt	Nucleotide
OD	Optical density
ONPG	*o*-nitrophenyl-β-D-galactopyranoside
ORF	Open reading frame
P	Proline
pA	Allele of the *Sip* gene which is associated with prolonged incubation period of SSBP/1 scrapie
pA80BR	Cheviot sheep brain cell culture from the negative line of the NPU flock, genotype ARQ/ARR
PAR	Proline alanine rich family of transcription factors
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
pH	$-\log_{10}$ (hydrogen ion concentration)
Phe	Phenylalanine
PIPLC	Phosphatidyl-inositol-specific phospholipase C
PK	Proteinase K
PKA	Protein kinase A
pmol	Picomoles
POD	Peroxidase
Prnd	Mouse doppel gene
Prnp	Prion protein gene
Prnp^a	Allele of the *Prnp* gene with 108L, 189T
Prnp^b	Allele of the *Prnp* gene with 108F 189V
Pro	Proline
PrP	Protease resistant protein / prion protein
PrP A	Mouse prion protein gene (*Prnp*) A allotype
PrP B	Mouse prion protein gene (*Prnp*) B allotype
PrP^c	PrP cellular form
PrP^{sc}	PrP scrapie, disease associated form
p7	Equivalent of the *Prnp*^{b} allele
PVDF	Poly vinylidene floride membrane
Py	Pyrimidine
Q	Glutamine
QTL	Quantitative trait loci
R	Arginine
RCLB	Reporter cell lysis buffer
RFLP	Restriction fragment length polymorphism
RNA	Ribose nucleic acid

RNase	Ribonuclease
rpm	Revolutions per minute
r/t	Room temperature
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
S	Serine
s7	Equivalent of the *Prnp*^{a} allele
sA	Allele of the *Sip* gene which is associated with short incubation period of SSBP/1 scrapie
SAF	Scrapie associated fibril
SAP	Shrimp alkaline phosphatase
sA80BR	Cheviot sheep brain cell culture from the positive line of the NPU flock, genotype VRQ/VRQ which is susceptible to SSBP/1 and some forms of natural scrapie but exhibits some resistance to BSE and CH1641
SCID	Severe combined immunodeficient mice
sCJD	Sporadic Creutzfeldt Jakob disease
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
^{Sec}PrP	Secreted PrP
Ser	Serine
sFI	Sporadic fatal insomnia
SHaPrP	Syrian hamster PrP
Sinc	Scrapie incubation time control gene in mice
Sip	Scrapie incubation time control gene in sheep
snRNPs	Small nuclear ribonuleoproteins
SOD	Superoxide dismutase
SP-1	Specificity protein-1
SSBP/1	Sheep scrapie brain pool 1
ssDNA	Single stranded DNA
STAT	Signal transducers & activators of transcription
STAT-A	STAT variant oligonucleotide
STAT-C	STAT wild type oligonucleotide
STE	Stop transfer effector domain
T	Threonine (amino acid) or thymine (nucleic acid), depending on context
TAE	Tris-acetate-EDTA electrophoresis buffer
TAFs	TATA-binding protein associated factors
TBE	Tris-boric acid-EDTA electrophoresis buffer
TBP	TATA-binding protein
TBS	Tris buffered saline
TBST	Tris buffered saline plus Tween 20 detergent
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylenediamine
TFIIB	General transcription factor
TFIID	Protein complex comprised of TBP and TAFs
TFIIE	General transcription factor
TFIIF	General transcription factor

TFIIH	General transcription factor
Thr	Threonine
TME	Transmissible mink encephalopathy
TNF-α	Tumour necrosis factor
Tris	Tris (hydroxymethyl) amino methane
Trp	Tryptophan
TSE	Transmissible spongiform encephalopathy
TVP	Trypsin/ versene solution
Tyr	Tyrosine
U	Uracil or Unit, depending on context
uAP-2	Upstream AP-2 oligonucleotide
µg	Microgram
µl	Microlitre
µM	Micromolar
µmol	Micromoles
UTR	Untranslated region
3'UTR	3' untranslated region
5'UTR	5' untranslated region
UV	Ultraviolet
V	Valine or volt, depending on context
Val	Valine
vCJD	Variant Creutzfeldt Jakob disease
v/v	Volume per volume
W	Tryptophan or Watt, depending on context
w/v	Weight per volume
X	Any amino acid
X-Gal	5-Bromo-4-Chloro-3-Indolyl-Beta-D-Galactopyranoside
Y	Tyrosine
YY1	Yin yang 1 transcription factor

Chapter 1: General introduction

1.1 Scrapie

Scrapie has been well documented throughout Europe since the 18th Century (Besnoit & Morel, 1898) and the first case of scrapie was reported in 1732 in England (McGowan, 1922). Scrapie was first described as a viral infection by Besnoit (1899) and scrapie infectivity was first demonstrated to be transmissible to other animals by Cuille & Chelle (1936). Scrapie is a naturally occurring slow degenerative disorder of the central nervous system (CNS) affecting both sheep and goats (Parry, 1962). It is a worldwide problem, however the disease is more prevalent in the Northern Hemisphere. Scrapie was eradicated by a rigorous slaughter policy in Australia and New Zealand (Brash, 1952), and following the introduction of extensive protection measures these countries are still recognised as being scrapie free. Most European countries have scrapie infected flocks, and the UK has averaged approximately 417 confirmed cases of scrapie a year over the last ten years (DEFRA, 2003). Other European countries with reported scrapie problems include Norway, the Netherlands, France, Iceland and the Republic of Ireland.

1.1.1 The transmissible spongiform encephalopathies (TSEs)

Scrapie is a member of a group of similar diseases, termed transmissible spongiform encephalopathies (TSEs) or prion diseases. This group of diseases include bovine spongiform encephalopathy (BSE) in cattle (Bradley, 2002), chronic wasting disease (CWD) of mule deer and elk in the USA (Laplanche, 1999, Miller, Williams, McCarty *et al.*, 2000), feline spongiform encephalopathy (FSE) in cats (Pearson, Wyatt, Gruffyddjones *et al.*, 1992), transmissible mink encephalopathy (TME) in captive mink (zu Rhein, Eckroade & Marsh, 1971) and Kuru, Gerstmann-Sträussler-Scheinker syndrome (GSS) and Creutzfeldt Jakob Disease (CJD) in man (Creutzfeldt, 1920, Gerstmann, 1928, Jakob, 1921, Zigas & Gajdusek, 1957) for a review see (Collinge, 1999). The TSEs are characterised by a loss of motor control, dementia (in human TSEs), paralysis and all are invariably fatal. The hallmark of TSEs is the aggregation into partially protease resistant fibrils of a pathological

isoform (PrP^{sc} for PrP scrapie) of a normal cellular protein designated (PrP^{c} for PrP cellular) (Prusiner, 1982). PrP^{c} is expressed normally in a variety of cellular tissues and is a soluble, membrane bound glycoprotein with a predominantly α-helical structure, which is sensitive to proteolytic digestion (Oesch, Westaway, Walchli *et al.*, 1985). In contrast, PrP^{sc} is a disease specific insoluble protein, which has a predominantly β-sheet structure and is partially resistant to proteolytic digestion (Oesch *et al.*, 1985). The detection of PrP^{sc} in *post mortem* tissue samples is used as a marker for disease, and other pathological markers for TSEs include vacuolation of brain tissue, which shows a distinct spongiform appearance and astrocytic gliosis (Bruce & Fraser, 1975). The PrP^{c} protein is expressed by the PrP gene, which is involved in the control of scrapie, as transgenic PrP null mice, which do not express endogenous PrP^{c}, exhibit resistance to scrapie infection (Manson, Jamieson, Baybutt *et al.*, 1999). In addition, polymorphisms within the PrP gene open reading frame (ORF) are known to be associated with differences in disease incubation period, and with disease susceptibility (Hunter, Moore, Hosie *et al.*, 1997, Moore, Hope, McBride *et al.*, 1998).

1.1.2 Scrapie clinical signs

Following scrapie infection of sheep an incubation period of between 2 and 5 years (depending on the scrapie strain) typically precedes the development of clinical signs. The behavioural signs of scrapie include nervousness, confusion and withdrawal from the flock. The physical signs of scrapie include an intense pruritis and affected sheep will often rub themselves against fence posts and bite at their own limbs or torso in an attempt to relieve this itching (Dickinson, 1976, Parry, 1962). This intensive scraping results in wool loss and skin abrasions particularly over the flanks and hindquarters and can last from between two weeks to six months, although the degree of pruritis can vary between individual animals and between breeds (Dickinson, 1976, Parry, 1962). Affected animals show signs of locomoter incoordination and ataxia, especially of the hind limbs which is accompanied by muscular tremors, resulting in the high stepping gait of the forelimbs often associated with scrapie affected animals. The appetite remains largely unaffected, however weight loss and emaciation can occur during the terminal weeks of the disease as the

animals eating and swallowing becomes impaired. After the onset of overt clinical signs most animals succumb to scrapie within one to three months, however some animals may survive for longer periods (Dickinson, 1976, Parry, 1962).

1.1.3 Scrapie diagnosis

At present there are no completely reliable tests available for the diagnosis of scrapie in live animals and definitive diagnosis is usually made *post mortem*. Histopathological examination of the CNS of scrapie affected sheep shows neurodegeneration with vacuolation of neurones, astrocytosis and characteristic spongiform changes (Bruce & Fraser, 1975). Diagnosis of scrapie may be confirmed by the detection of scrapie associated fibrils (SAFs) using biochemical purification by centrifugation and visualisation by electron microscopy (Rubenstein, Merz, Kascsak *et al.*, 1987). These amyloid fibrils can be isolated from brain tissue of scrapie-infected animals and consist of aggregates of PrP^{sc} (Rubenstein *et al.*, 1987). PrP^{sc} is more reliably detected by Western blot or by immunohistochemistry (IHC) using antibodies specific to prion protein. However, in order to detect PrP^{sc} specifically in tissue samples, the tissue must be treated with proteinase K to degrade the normal (proteinase K sensitive) isoform of prion protein (PrP^{c}) prior to immunodetection.

In recent years a number of high throughput post mortem diagnostic tests have been developed, which detect PrP^{sc} using a range of specific antibodies. In addition, it was noticed that some of these tests were able to diagnose a TSE infection at least three months prior to the development of clinical signs (Grassi, Comoy, Simon *et al.*, 2001). Methods of ante mortem detection have been developed and these include detection of PrP^{sc} in tonsillar tissue (Schreuder, van Keulen, Vromans *et al.*, 1998) and third eyelid tissue in sheep over 14 months of age (O'Rourke, Baszler, Parish *et al.*, 1998), however, these are not completely reliable. The holy grail of ante mortem TSE detection remains a rapid, sensitive and reliable blood test capable of detecting the presence of PrP^{sc} prior to the development of clinical signs (Grassi, 2003). However, this is currently hampered by the fact that the level of infectivity detectable in the blood of infected animals is considerably lower than the levels found in, for example, the CNS (Grassi, 2003). Recent studies have

shown that it may be possible to amplify the levels of PrP^{sc}, thus making a test based on blood more feasible (Saborio, Permanne & Soto, 2001). PrP^{sc} has been detected in urine samples from animals and humans, raising the possibility of a urine based TSE test (Shaked, Shaked, Kariv-Inbal *et al.*, 2001).

Paramithiotis, Pinard, Lawton *et al.* (2003) successfully produced both polyclonal and monoclonal antibodies which are able to distinguish between PrP^{c} and PrP^{sc}. Their antibodies are targeted to a tripeptide motif (Tyr-Tyr-Arg), which is considered to be hidden in PrP^{c} but exposed upon conversion to PrP^{sc}. Characterisation of these antibodies revealed that most of them showed cross reactivity with PrP^{sc} from mice, hamster, sheep, cattle and humans. It is hoped that these antibodies will help to further develop TSE diagnostics and could also have potential therapeutic applications. However these antibodies are not completely specific for PrP^{sc} as they also immunoprecipitate partially denatured protease sensitive PrP from acid treated healthy brain and this may indicate that they are able to detect other alternative conformations of PrP. In addition, these antibodies are directed to Tyr-Tyr-Arg motifs it is possible that they may also react with other proteins which contain the same motif (Gorochov & Deslys, 2004).

Although, the presence of PrP^{sc} is the hallmark of prion diseases, other biochemical factors have been identified as potential markers for TSE diagnosis. These include the presence of elevated levels of neuron specific enolase (NSE) and 14-3-3 proteins in the cerebrospinal fluid (CSF) in patients suffering from CJD (Hsich, Kinney, Gibbs *et al.*, 1996, Mokuno, Kato, Kawai *et al.*, 1983, Zerr, Bodemer, Otto *et al.*, 1996, Zerr, Bodemer, Racker *et al.*, 1995, Zerr, Bodemer & Weber, 1997). These factors are not specific for TSEs and are often elevated in other neurological diseases, however they do provide an additional diagnostic tool, which can be used in conjunction with other clinical data to improve diagnosis (Weber, Otto, Bodemer *et al.*, 1997). More recently, Miele, Manson & Clinton (2001) showed that the expression of a transcript specific to erythroid linaege cells is dramatically decreased in TSEs. The measurement of this transcript can be made in a number of easily accessible tissues including bone marrow, spleen and whole blood, indicating a possible role for this marker in TSE diagnosis.

1.1.4 Scrapie Control

Historically scrapie has been controlled by the identification and culling of scrapie infected animals and sometimes entire flocks. However, this method can be unreliable as it depends on the successful identification of infected animals, and is very costly. Scrapie control is hampered by the fact that as yet no reliable pre-clinical scrapie test exists, and the long incubation periods associated with the disease. In recent years the British government has introduced the National Scrapie Plan (NSP), the aim of which is to increase the level of resistance to scrapie in the national sheep flock by selectively breeding for animals with recognised scrapie resistant PrP genotypes (DEFRA, 2003). The long-term aim of this plan is to completely eradicate scrapie and other TSEs from the national flock (Dawson, Warner, Nolan *et al.*, 2003).

1.1.5 The pathogenic mechanisms of TSEs

The principal cause of the clinical symptoms observed with TSEs is the death of neurones (Giese, Groschup, Hess *et al.*, 1995, Jamieson, Jeffrey, Ironside *et al.*, 2001, Kretzschmar, Giese, Brown *et al.*, 1997, Lucassen, Williams, Chung *et al.*, 1995, Williams, Lucassen, Ritchie *et al.*, 1997). There is evidence that this neuronal cell death is the result of apoptosis, as signs of programmed cell death have been discovered by numerous researchers in a variety of models (Ferrer, 1999, Forloni, Bugiani, Tagliavini *et al.*, 1996, Jamieson *et al.*, 2001, Jesionek-Kupnicka, Buczynski, Kordek *et al.*, 1997, Kretzschmar *et al.*, 1997). The apoptosis of neurones observed in TSEs is the result of a variety of influences, one of which is an increase in oxidative stress which is brought about by the accumulation of reactive oxygen species (Beal, 1995, Gotz, Kunig, Riederer *et al.*, 1994). However, altered cellular calcium metabolism, has been observed in TSEs, which could associate necrosis as a contributing mechanism in neurodegeneration in TSEs (Giese *et al.*, 1995, Lucassen *et al.*, 1995). Other potential causative factors include, mitochondrial dysfunction, altered iron metabolism leading to the production of highly reactive hydroxyl radicals, and increased levels of proinflammatory cytokines, chemokines and transcription factors, like nuclear factor-kappa B (NF-κB) (Allen & Tresini, 2000,

Choi, Ju, Choi *et al.*, 1998, Hur, Chang, Lee *et al.*, 2001, Kaltschmidt, Baeuerle & Kaltschmidt, 1993, McGeer & McGeer, 1995). Ma, Wollmann, & Lindquist (2002) demonstrated that misfolded PrP^{c} is itself neurotoxic in both cultured cells and transgenic mice. The accumulation of misfolded PrP^{c} in the cytosol resulted in cerebellar degeneration, gliosis and eventually a severe ataxia in transgenic mice, in a similar way to that observed with PrP^{sc} (Ma *et al.*, 2002).

1.1.6 TSEs of humans

TSEs of humans can be divided into those that are inherited, sporadic or iatrogenic in nature. Inherited TSEs include GSS, fatal familial insomnia (FFI) and familial CJD (fCJD), and all of these diseases are associated with variants of the PrP protein. The factors involved in familial CJD remain unclear but the disease shows an autosomal dominant pattern of transmission, similar to Alzheimer's disease (Masters, Gajdusek & Gibbs, 1981, Masters, Harris, Gajdusek *et al.*, 1979, Prusiner, 1993). Patients with fCJD show early signs of dementia and visual impairment, the duration of disease is between 2-5 months and age of onset is generally between 50-75 years but these signs may vary from case to case (Prusiner, 1993). GSS appears to be the result of a missense mutation which substitutes proline for leucine at codon 102 within the human PrP gene (Collinge, Harding, Owen *et al.*, 1989, Dohura, Tateishi, Sasaki *et al.*, 1989, Hsiao, Baker, Crow *et al.*, 1989). GSS is set apart from the other human TSEs as it tends to have a much longer duration of disease, normally between 2-8 years. The age of onset is generally in middle age at between 35-55 years of age, early signs are those of ataxia and this develops into full dementia as the disease progresses (Prusiner, 1993).

Sporadic TSEs are limited to sporadic CJD (sCJD), which is a rare condition with only 1-2 cases per million worldwide. The mean age at death of sCJD is 63 years of age and the duration of illness is usually around 4 months (Prusiner, 1993).

Iatrogenic CJD, variant CJD (vCJD) and Kuru are all infectious diseases which are acquired by transplantation, injection or ingestion of TSE infected material (Chazot, Broussolle, Lapras *et al.*, 1996, Gajdusek, 1977, Pfeffer, 1993, Will, Ironside, Zeidler *et al.*, 1996). Kuru was first described by Zigas & Gujdusek (1957), and is restricted to the Fore tribe in Papau New Guinea's Eastern Highlands

(Gajdusek, 1963, Gajdusek, 1973). Kuru is the result of ritualistic cannibalism of dead relatives, which occurred as part of the mourning ritual, almost exclusively amongst the female members of the tribe as a direct result of their involvement in the preparation of dead bodies for disposal (Gajdusek, 1963, Gajdusek, 1973). Kuru is characterised by ataxia and a shivering like tremor, that later progresses to motor incapacity, loss of speech and inevitably death, usually after 3-9 months (Zigas & Gajdusek, 1957). vCJD was first described by Will *et al.* (1996) when it was discovered that a small number of cases of CJD had occurred in young adults (mean age of 29 at death) referred to the National CJD Surveillance Centre in Edinburgh. The average duration of disease in vCJD is longer than that observed with fCJD at around 13 months (Will *et al.*, 1996). It was noted that these cases showed a distinct clinical and pathological phenotype that differed from that observed with sporadic CJD. Will *et al.* (1996) suggested that these cases of vCJD may have been linked to the UK epidemic of BSE in cattle. This theory is supported by studies which have shown distinct similarities between the PrP^{sc} deposited in vCJD and experimental BSE cases (Collinge, Sidle, Meads *et al.*, 1996). In addition, transmission studies in mice have demonstrated that vCJD and BSE share certain disease characteristics, such as incubation period and distribution of pathology in the brain, and that these characteristics are distinct from those observed with sporadic CJD (Hill, Desbruslais, Joiner *et al.*, 1997, Scott, Will, Ironside *et al.*, 1999). There is now little doubt that vCJD is caused by BSE infection.

1.1.7 Other TSEs of animals

The most well documented TSE of animals is the epidemic of BSE in cattle in the UK from 1986. However, BSE has been diagnosed in cattle in many other European countries, including France, Portugal, Germany, Italy, Ireland and Switzerland, although almost all of these cases were probably due to cattle or feed products imported from the UK (Bradley, 2002, Wells, Scott, Johnson *et al.*, 1987). It is believed that BSE in the UK occurred due to a change in rendering procedures in the late 1970s and early 1980s that allowed feed products to become contaminated with infected material, and that the feeding of this contaminated feed to cattle led to the outbreak of BSE (Bradley & Matthews, 1992, Wilesmith, Wells, Cranwell *et al.*,

1988). What is less clear is how the infected material entered the cattle food chain in the first place. It could have been that it occurred sporadically in cattle and that tissue from this animal entered the rendering process or that it developed from scrapie infected material (Anil, Love, Williams *et al.*, 1999).

BSE is characterised by an incubation period of between 2-8 years and most affected cattle develop clinical signs of the disease between 3-5 years of age (Bradley, 2002). Clinical signs are those of incoordination, tremors and anxiety, and death usually follows between two weeks and 6 months after the development of these clinical signs (Bradley, 2002). A ban on the feeding of ruminant material to other ruminants was introduced in 1988 in the UK, however due to the long incubation periods of the disease and difficulties in enforcement of the ban a further 42,299 cases of BSE (born after the ban (BABs)) occurred after this initial ban (Bradley & Wilesmith, 1993). Further, more stringent bans were introduced between 1988 and 1996, and finally in August 1996 a total ban (termed the real ban) on the feeding of meat and bone meal to any food animal species was introduced. Since this ban few cases of BSE (born after the real ban (BARBs)) have been reported in the UK, compared with a total of 187,870 cases of BSE in the UK in total (Bradley, 2002).

Chronic wasting disease (CWD) is a prion disease of both wild and captive deer and elk in North America (Laplanche, 1999, Miller *et al.*, 2000). Transmissible mink encephalopathy (TME) of captive mink in fur producing farms is believed to be caused by contaminated feed, however the source of the food contamination remains unclear (Hartsough & Burger, 1965, zu Rhein *et al.*, 1971). A number of other prion diseases affecting animals have been reported, for example, feline spongiform encephalopathy (FSE) of zoological and domestic cats, in addition, sporadic cases of TSE have occurred in zoological ruminants and non-human primates (Jeffrey & Wells, 1988, Kirkwood, Wells, Wilesmith *et al.*, 1990, Pearson *et al.*, 1992, Wyatt, Pearson, Smerdon *et al.*, 1991). The origins of the TSEs in cats, zoological ruminants, and non-human primates are most likely linked to the feeding of BSE contaminated meat.

1.2 The nature of the infectious agent

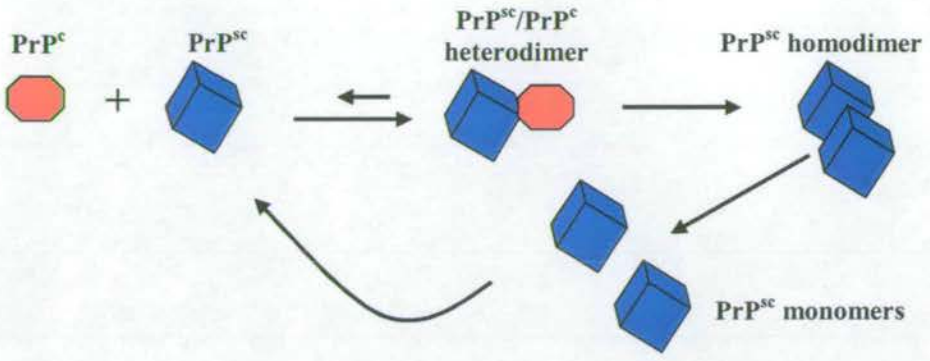
The nature of the scrapie infectious agent has been the subject of intense research for many years. The infectious agent causing scrapie was described as an unconventional slow virus by Sigurdsson (1954) whilst working on scrapie and visna of sheep in Iceland. Similar observations were made by Hadlow (1959) who made the connection between scrapie and Kuru in New Guinea; concluding that Kuru was also caused by a slow virus. It was observed that the scrapie agent was resistant to inactivating procedures such as intense ultraviolet light and ionising radiation that inactivate or modify nucleic acids (Latarjet & Muel, 1970). It was observed that the agent was sensitive to treatments that denature, hydrolyse or degrade proteins (Bolton, McKinley & Prusiner, 1982, McKinley, Bolton & Prusiner, 1983). These studies indicated that the agent could be a self-replicating protein, which was devoid of nucleic acids (Alper, Cramp, Haig *et al.*, 1967, Griffith, 1967, Latarjet & Muel, 1970). Many methods of inactivation of the scrapie infectious agent have been tested, examples of techniques tested so far, include autoclaving, acid, alkaline, oxidising agents, detergents and sodium hypochlorite (Taylor, 2000). Combinations of these methods, for example, autoclaving carried out in alkaline solutions have been shown to inactivate the scrapie agent (Brown, Rohwer, Green *et al.*, 1982, Taylor, Fernie & McConnell, 1997). In addition, a strong solution of sodium hypochlorite has been shown to completely inactivate the scrapie infectious agent (Taylor, 2000).

1.2.1 The prion hypothesis

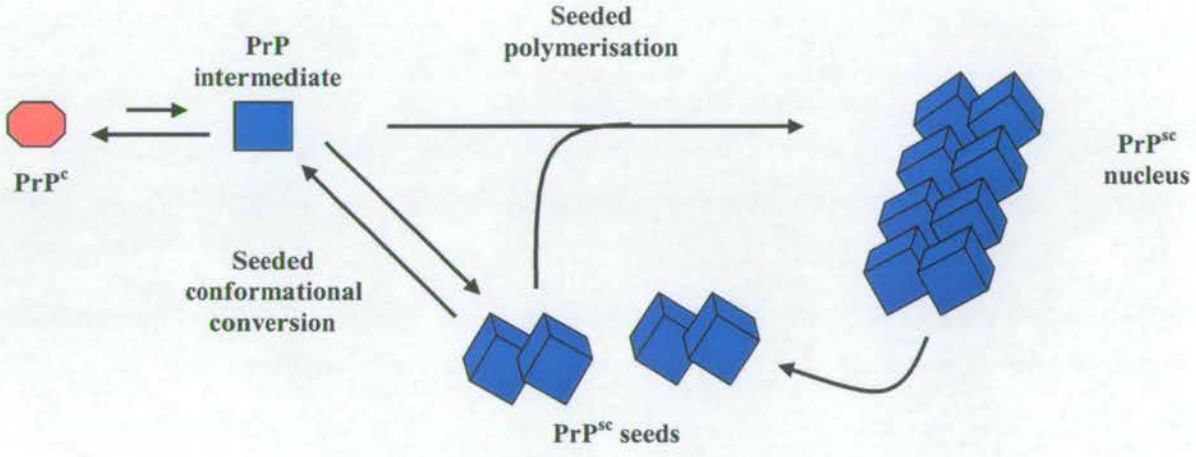
There are two major hypotheses on the nature of the infectious agent, the prion hypothesis and the virino hypothesis. Prusiner (1982) hypothesised that the scrapie infectious agent consisted of a novel class of infectious agent which he termed, prions (proteinaceous infectious particles). Prusiner, Groth, Bolton *et al.* (1984) used proteinase K digestion and differential centrifugation to purify the infectious agent from scrapie infected hamster brains. Rod shaped amyloid particles were shown to co-purify with scrapie infectivity as measured in bioassays, and were composed largely of a single protein of between 27-30 kDa (PrP 27-30), a protease resistant fragment of a 33-35 kDa precursor termed PrP^{sc} (Hope & Hunter, 1988,

Prusiner, 1982, Prusiner *et al.*, 1984). Further studies identified PrP^{sc} as an isoform of PrP^{c}, a protein encoded by a cellular gene, and therefore not a virally encoded protein (Basler, Oesch, Scott *et al.*, 1986, Chesebro, Race, Wehrly *et al.*, 1985, Oesch *et al.*, 1985).

The absence of PrP^{sc} in the brains of healthy individuals combined with its presence in the brains of individuals infected with most of the TSE diseases, suggests that PrP^{sc} is either itself the infectious agent, or a component of it, and as such the accumulation of PrP^{sc} is considered to be a biochemical hallmark of the TSEs. The prion hypothesis proposes that PrP^{sc} is the infectious agent and that it is able to replicate devoid of any nucleic acid by converting the endogenous PrP^{c} to PrP^{sc} (Griffith, 1967, Harris, 1999, Prusiner, 1982, Prusiner, 1991, Prusiner, 1998, Prusiner, Scott, Foster *et al.*, 1990). Two models have been proposed for the conversion of PrP^{c} to PrP^{sc}, the conformational conversion model (Figure 1.1A) and the nucleated polymerisation model (Figure 1.1B).



A. *Conformational conversion model*



B. *Nucleated polymerisation model*

Figure 1.1 *Proposed models for the conversion of PrP^{C} to PrP^{SC}*

There are two proposed models for the conversion of PrP^{C} to PrP^{SC}. The conformational conversion model (A) hypothesises that PrP^{C} & PrP^{SC} interact forming a heterodimer, and then the PrP^{C} in this heterodimer is converted to PrP^{SC} forming a PrP^{SC} homodimer, which is able to dissociate, producing PrP^{SC} monomers. The nucleated polymerisation model (B) hypothesises that PrP^{C} is converted to PrP^{SC} when PrP^{SC} is present at a concentration that favours the conversion of a PrP intermediate into a PrP^{SC} oligomeric molecule. Models adapted from (Prusiner, 1991) & (Jarrett & Lansbury, 1993).

The conformational conversion model proposes that PrP^{C} and PrP^{SC} interact directly to form a heterodimer, PrP^{C} is then converted to PrP^{SC} to form a PrP^{SC}

homodimer. This homodimeric PrP^{sc} is then able to dissociate, producing two PrP^{sc} monomers, which can go on to convert more PrP^{c} to PrP^{sc} (Prusiner, 1991). The nucleated polymerisation model proposes that PrP^{c} is converted to PrP^{sc} when PrP^{sc} is present at a concentration that favours the conversion of a PrP intermediate into a PrP^{sc} oligomeric molecule (Jarrett & Lansbury, 1993). Eigen (1996) performed a comparative kinetic analysis of the conformational conversion and nucleated polymerisation models and concluded that although both models differ as to which of the two monomeric protein conformations are favoured at the equilibrium state (i.e. PrP^{c} and PrP^{sc} for the conformational conversion model, and a PrP intermediate for the nucleated polymerisation model) they both require an aggregated state as the form which is favoured at equilibrium.

A prion infection is caused by the passage of PrP^{sc} from an infected host to a recipient animal and this PrP^{sc} molecule then initiates conversion of endogenous PrP^{c} to PrP^{sc} in the recipient (Prusiner, 1991). The major arguments in support of the prion hypothesis are detailed below:

- Prion diseases are invariably associated with the accumulation of PrP^{sc} and amyloid plaques (Bolton *et al.*, 1982, Oesch *et al.*, 1985, Prusiner, Bolton, Groth *et al.*, 1982).
- PrP^{sc} and scrapie infectivity co-purify, the unusual properties of PrP^{sc} mimic those of prions and procedures that modify or hydrolyse PrP^{sc} also inactivate prions (Gabizon, McKinley, Groth *et al.*, 1988, Prusiner, 1982, Prusiner *et al.*, 1984).
- Certain mutations in the PrP gene are linked to inherited prion diseases and result in the formation of PrP^{sc} (Goldfarb, Petersen, Tabaton *et al.*, 1992, Hegde, Mastrianni, Scott *et al.*, 1998a, Hsiao, Cass, Schellenberg *et al.*, 1991).
- PrP null mice are resistant to prion infection, whilst heterozygotes show an increase in incubation period, as compared to wild type mice (Bueler, Fischer, Lang *et al.*, 1992, Manson, Clarke, Hooper *et al.*, 1994a, Manson, Clarke, McBride *et al.*, 1994b, Weissmann, Bueler, Fischer *et al.*, 1994).
- Prion diversity is enciphered within the conformation of PrP^{sc}. Therefore, scrapie strains can be maintained by passage through hosts with different PrP genes.

Prion strains are themselves maintained by PrP^{c}/PrP^{sc} interactions (Bessen, Kocisko, Raymond *et al.*, 1995, Bessen & Marsh, 1994, Caughey, Raymond & Bessen, 1998, Ridley & Baker, 1996, Safar, Wille, Itrri *et al.*, 1998, Telling, Parchi, DeArmond *et al.*, 1996).

- Protein based instructional (informational) molecules exist. Prion-like molecules (i.e. [URE3], [PSI] and [Het-s*]) have been discovered in yeast and fungi that appear to be responsible for the propagation of non-genetic traits (Chernoff, Lindquist, Ono *et al.*, 1995, Coustou, Deleu, Saupe *et al.*, 1997, Cox, 1994, Wickner, 1994, Wickner, Edskes, Maddelein *et al.*, 1999).
- Currently no evidence for a virus like particle or a nucleic acid specifically associated with TSE infectivity exists.

1.2.2 The virino hypothesis

The virino theory postulates that PrP^{sc} is a component of the infectious agent, which is recruited into an infectious particle by an as yet unidentified host-independent molecule, likely to be viral nucleic acid (Dickinson & Outram, 1988, Farquhar, Somerville & Bruce, 1998). The virino hypothesis postulates that the information-carrying molecule of the infectious agent interacts with host PrP^{c} and is protected from degradation by the conversion of PrP^{c} to PrP^{sc} (Farquhar *et al.*, 1998). Attempts to identify a TSE-specific nucleic acid have proved unsuccessful, however, it may be that the molecule involved is either too small or present at too low a concentration to be detectable (Bruce & Dickinson, 1987, Somerville, 1991).

Although the accumulation of PrP^{sc} is associated with the development of disease, several studies have demonstrated neurological disease in mice in the absence of PrP^{sc}. Lasmezas, Deslys, Robain *et al.* (1997) inoculated C57BL/6 mice with BSE infected cattle brain homogenate. All the mice showed symptoms of neurological disease following incubation periods of between one and two years. Intriguingly, more than 55% of the mice failed to show detectable levels of PrP^{sc} in their brains, however PrP^{sc} was detectable following subsequent sub-passages in mice as the agent appeared to adapt to its new host. This study indicated that TSE infectivity could exist in the absence of detectable levels of PrP^{sc} and that another unidentified agent may therefore be responsible for disease transmission.

Other studies have demonstrated the presence of detectable PrP^{sc} in the absence of clinical disease symptoms. It is generally accepted that mice are highly resistant to infection with hamster scrapie, however transgenic mice expressing chimeric PrP^{c} in which the central region (residues 94-188) of the mouse PrP has been replaced with the corresponding hamster sequence (termed MH2M PrP^{c}), are susceptible to infection with hamster scrapie (Scott, Groth, Foster *et al.*, 1993). Hill, Antoniou & Collinge (1999) used this property to convert the chimeric MH2M PrP^{c} to MH2M PrP^{sc} *in vitro* by using hamster PrP^{sc}, thus any *de novo* MH2M PrP^{sc} would be easily distinguished from the hamster PrP^{sc} used to promote the conversion. The MH2M PrP^{sc} produced was then used in bioassay experiments in conventional mice, however, no infectivity was detected in the inoculated mice, indicating that the accumulation of PrP^{sc} is by itself not sufficient for the propogation of infectivity (Hill *et al.*, 1999). In further experiments, Hill, Joiner, Linehan *et al.* (2000) inoculated Swiss CD-1 mice with hamster prions and showed that they propagate infectivity, produce detectable levels of PrP^{sc}, and develop typical spongiform degeneration and amyloid deposition in their brains. However, all of these mice remained free of clinical disease for the duration of their lifetime.

These studies have demonstrated the existence of sub-clinical forms of TSE disease and indicate that PrP^{sc} alone may not be sufficient as a marker for disease, or infectivity.

The major arguments in support of the virino hypothesis are detailed below:

- The presence of multiple TSE strains is more clearly explained by the virino hypothesis as it is well documented that viral nucleic acids are capable of carrying strain-specific information (Dickinson & Outram, 1988).
- The estimated size of the infectious agent, as determined by ionising radiation falls within the range observed with certain viral nucleic acid genomes (1.5×10^{6} Da or 0.90×10^{6} Da), depending on whether or not the genome is composed of double stranded or single stranded DNA (Rohwer, 1991).
- Diringer, Beekes, Ozel *et al.* (1997) noted the presence of virus-like particles in TSE affected brains, but this observation has not been repeated by other researchers.

- Finally the prion hypothesis remains unproven, as infectivity has still not been produced *de novo* by experimental manipulation of recombinant or synthetic PrP.

1.3 TSE strains

The number of strains occurring in species naturally affected by scrapie is unknown, however in mice approximately twenty different scrapie strains have been identified (Bruce, McBride, Jeffrey *et al.*, 1994). Scrapie strains were first identified in goats, with two distinct strains being present, named scratching and drowsy based on their differing clinical signs (Pattison & Millson, 1961). Each strain is described by characteristic neuropathological patterns of PrP^{sc} protein deposition and vacuolation as well as varying incubation periods i.e. defined by the time elapsed between the initial infection and clinical signs of disease (Bruce & Fraser, 1991, Bruce *et al.*, 1994, Fraser, 1976). Recent studies by Gonzalez, Martin, Begara-McGorum *et al.* (2002) produced profiles of PrP^{sc} deposition in the brains of 43 sheep with clinical signs of scrapie using IHC. They concluded that these profiles could be successfully used to characterise different scrapie strains in sheep. For the most part, each individual strain has been shown to retain its original properties when propagated within the same species, between species or in cultured cells (Birkett, Hennion, Bembridge *et al.*, 2001, Gabizon & Taraboulos, 1997, Raymond, Hope, Kocisko *et al.*, 1997).

The molecular heterogeneity of PrP^{sc} is another characteristic used to identify strains. PrP^{sc} retains a strain characteristic glycosylation pattern when visualised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Collinge *et al.*, 1996). This observation led to the conclusion that the attached carbohydrate moieties of PrP^{sc} were able to encipher strain-specific properties. This idea has since been dismissed as the glycosylation pattern of PrP^{sc} purified from different tissues within a single host has been shown to differ, suggesting that glycosylation of PrP is not determined by the strain but by the cell type and species in which it is produced (Hill *et al.*, 1999, Somerville, 1999). However, more recently Pan, Colucci, Wong *et al.* (2001) using two dimensional electrophoresis were able to show that the only conformational difference between two PrP^{sc} species (sporadic fatal insomnia (sFI) and sporadic CJD (M/M2 sCJD)) were those determined by the variant glycans.

Therefore, this study showed that two PrP^{sc} species associated with distinct phenotypes differed only in the attached glycan chains.

The presence of different strains of scrapie indicates that the infectious agent must contain, or be associated with some form of information-carrying molecule (Farquhar *et al.*, 1998). The prion hypothesis states that scrapie strains are encoded in the conformational state of individual PrP^{sc} molecules, variations in the tertiary structure of PrP^{sc} would therefore be capable of enciphering the information required for strain-specific characteristics (Bessen *et al.*, 1995, Bessen & Marsh, 1994, Caughey *et al.*, 1998, Ridley & Baker, 1996, Safar *et al.*, 1998, Telling *et al.*, 1996). Fourier transform infrared (FTIR) spectroscopy studies, which showed that PrP^{sc} from various hamster-adapted scrapie isolates exhibits differences in the degree of β-sheet content support the prion hypothesis (Caughey *et al.*, 1998). The virino theory helps to explain scrapie strain characteristics, as a nucleic acid component would be capable of carrying the required genetic information (Somerville, 1991).

1.4 Scrapie transmission

1.4.1 Ovine transmission

Although the natural route of scrapie transmission remains unclear, vertical transmission has been hypothesised as a possible route of infection. Dickinson, Stamp & Renwick (1974) showed that lambs born to ewes infected with scrapie were seven times more likely to develop disease than those born to scrapie free ewes. Studies on maternal transmission using embryo transfer have investigated whether disease can be transmitted early in gestation or in the ovary, by transferring embryos from ewes infected with scrapie into recipient ewes shown to be clinically free from scrapie (Foote, Clark, Maciulis *et al.*, 1993, Foster, Hunter, Williams *et al.*, 1996, Foster, McKelvey, Mylne *et al.*, 1992). However, the results from these studies have been inconclusive, Foote *et al.* (1993) showed that scrapie was not transmissible via embryo transfer, whilst studies by Foster *et al.* (1996, 1992) showed that scrapie developed in embryos from both infected and uninfected hosts. In addition, the placenta has been shown to harbour infectivity, indicating that the placenta may play an important role in the natural transmission of scrapie (Andreoletti, Lacroux,

Chabert *et al.*, 2002, Race, Jenny & Sutton, 1998). Therefore, the placenta could be considered as a source of scrapie infectivity to newborn lambs, or at the time of birth, in addition, it could be considered as a source of horizontal transmission to other sheep if consumed directly or through pasture infection (Andreoletti *et al.*, 2002).

In fact, horizontal transmission has been demonstrated between scrapie infected sheep and healthy sheep and goats when housed together, although the exact mechanism of transmission remains unclear (Brotherston, Renwick, Stamp *et al.*, 1968, Haralambiev, Ivanov, Vesselinova *et al.*, 1973). More recent studies have looked at the possibility of transmission of infectivity through blood (Houston, Foster, Chong *et al.*, 2000, Hunter, Foster, Chong *et al.*, 2002). They showed that, at the time of publication, 2 out of 24 sheep transfused with blood from BSE challenged sheep developed the disease, whilst 4 of 21 sheep transfused with blood from natural scrapie infected sheep developed scrapie, and more have since occurred (Houston *et al.*, 2000, Hunter *et al.*, 2002). These results indicate that BSE and scrapie could certainly be transmitted through blood, or blood products, and this has serious implications for human blood transfusions with regard to vCJD (Houston *et al.*, 2000, Hunter *et al.*, 2002). Attempts to detect PrP^{sc} and infectivity in buffy coat from vCJD patients have proved to be negative, however these studies were limited by sample numbers, and more importantly by the sensitivity of the detection methods used. Further studies will be required in order to develop more sensitive techniques for the detection of PrP^{sc} in blood, and blood products (Ironside & Head, 2003). The issue of blood borne infectivity is highlighted by the case of a UK patient who died last autumn of vCJD, which he may have contracted from a blood transfusion he received from a donor who died of vCJD back in 1999 (Llewelyn, Hewitt, Knight *et al.*, 2004).

Epidemiological evidence from studies on scrapie management in Iceland had suggested that it was possible that in some instances, a vector could be involved in the reoccurrence and/or spread of scrapie (Palsson & Sigurdsson, 1959). The possible involvement of a vector in scrapie is supported by the absence of scrapie in some countries, i.e. New Zealand & Australia (Brash, 1952). Indicating that in these countries the vector may be absent and therefore the disease is unable to propagate even in the presence of animals with known scrapie susceptible genotypes. The

involvement of a vector has been investigated by a number of researchers in the past and possible vectors identified include, hay mites or other mites species, and nematodes (Fitzsimmons & Pattison, 1968, Wisniewski, Sigurdarson, Rubenstein *et al.*, 1996). However, these studies have been limited in number, and the results have been inconclusive and often confused by the presence of other infections.

1.4.2 The species barrier

It is hypothesised that polymorphisms in the PrP gene may be responsible for control of the species barrier. This is a phenomenon that is observed if an infectious inoculum from one species (i.e. hamster) is passaged into a different species (i.e. mice), resulting in a prolongation of incubation period, or complete lack of clinical signs (Farquhar *et al.*, 1998). Another feature of the species barrier is that on subsequent serial sub-passage in a new host the incubation period shortens to a constant duration (Dickinson, 1976, Pattison & Jones, 1968). For example, the Chandler mouse-passaged strain of scrapie is transmissible to mice with an incubation period of around 120 days, whilst transmission to Syrian hamsters is possible, but with an incubation period of up to 380 days (Kimberlin, Cole & Walker, 1987, Kimberlin & Walker, 1978). Furthermore, the hamster-passaged scrapie strain, 263K, fails to transmit to mice, despite short incubation periods of around 65 days observed with hamster infections (Kimberlin, Walker & Fraser, 1989). There are, however, situations where scrapie can be transmitted into another species with no apparent species barrier effect, for example the transmission of scrapie from sheep to goats (Brotherston *et al.*, 1968, Greig, 1950).

1.4.2.1 Transgenic analysis of the species barrier

Transgenic studies have allowed the idea of the species barrier to be investigated further, for example, Scott *et al.* (1993) constructed transgenic mice which expressed chimeric mouse/Syrian hamster PrP (SHaPrP) genes. These mice were susceptible to infection with both Syrian hamster and mouse prions, and upon *post mortem* examination of the brains, chimeric PrP^{sc} was observed (Scott *et al.*, 1993). It was shown that this chimeric PrP^{sc} favoured propagation in mice expressing

the corresponding chimeric PrP^c (Scott *et al.*, 1993). In addition, the infection of transgenic mice expressing the hamster PrP gene resulted in the development of disease when infected with hamster scrapie, whilst animals expressing only endogenous PrP^c did not become infected (Race, Priola, Bessen *et al.*, 1995, Scott, Foster, Mirenda *et al.*, 1989). These results provide evidence for homophilic interactions between PrP^{sc} in the inoculum and host PrP^c and highlight the importance of the primary structure of PrP^c encoded by the host in the species barrier (Prusiner *et al.*, 1990, Scott *et al.*, 1989, Scott *et al.*, 1993, Scott, Groth, Tatzelt *et al.*, 1997, Scott, Kohler, Foster *et al.*, 1992, Westaway, Goodman, Mirenda *et al.*, 1987).

More recently Peretz, Williamson, Legname *et al.* (2002) inoculated transgenic mice expressing a chimeric mouse/ hamster PrP (TgMH2M), and transgenic mice expressing hamster PrP with two distinct Syrian hamster prion strains, Sc237 & DY. They then assessed the incubation time and phenotype of the disease produced in the hosts and correlated this information with data on the conformation of the two strains obtained. Following inoculation in TgMH2M mice the two strains, Sc237 and DY, behaved very differently. The Sc237 strain showed an incubation period of around 83 days, which decreased to around 50 days on second passage, indicating a species barrier effect. However, the TgMH2M mice inoculated with the DY strain showed no reduction in incubation period following second passage, a result consistent with a lack of species barrier. The disease phenotype observed with the Sc237 strain differed from that observed with Sc237 in hamsters, whilst that observed with the DY strain remained the same. These results were interpreted to indicate that the structures of the two strains are different, and that the conversion of the Sc237 strain into a distinct isoform in the TgMH2M mice was the result of a conformational change, whilst with the DY strain no such change was detected (Peretz *et al.*, 2002).

1.4.2.2 BSE and the species barrier

There is now a strong body of evidence supporting the transmission of BSE to humans in the form vCJD (Will *et al.*, 1996). The appearance of vCJD was first noted in 1996, occurring mostly in young adults (Chazot *et al.*, 1996, Will *et al.*, 1996). Similarities between the BSE agent and that causing vCJD were later

discovered by lesion profile and glycoform analysis (Bruce, Will, Ironside *et al.*, 1997, Collinge *et al.*, 1996, Hill *et al.*, 1997). These similarities have been reflected in transgenic analysis of BSE and vCJD transmissions. Transgenic mice expressing the bovine PrP gene were infected with primary passage BSE and vCJD and showed a high degree of susceptibility to both agents (Scott *et al.*, 1999). Interestingly, incubation periods, neuropathology and Western blot analysis of PrP^{sc} failed to discriminate between the two agents (Scott *et al.*, 1999). These studies have cast doubts over the earlier assumptions that humans were in some way protected from BSE infection from cattle due to the presence of a species barrier and it now appears that the infectious agent causing BSE may be capable of infecting new host species.

1.4.2.3 BSE in sheep?

The possibility that BSE could be present in small ruminants has remained a concern ever since Foster, Hope & Fraser (1993) first reported the experimental transmission of BSE to sheep and goats. This study involved orally challenging Cheviot sheep from the NPU flock with 0.5g BSE infected cattle brain. Only a proportion of the challenged sheep succumbed to disease, which was later shown to be related to the PrP genotype. The shortest incubation periods were observed in sheep of genotypes AXQ/AXQ, whilst animals with one ARR allele showed very extended incubation periods (Foster *et al.*, 1993, Foster, Parnham, Chong *et al.*, 2001, Houston, Goldmann, Chong *et al.*, 2003). For an explanation of sheep PrP genotypes please see section 1.7.2. More recent experiments showed that Romney sheep of the genotype ARQ/ARQ were susceptible to oral BSE challenge (Jeffrey, Ryder, Martin *et al.*, 2001). In addition, subtle differences were observed in the peripheral pathogenesis of a BSE infection in sheep when compared to that of natural scrapie infection (Jeffrey *et al.*, 2001). In particular, the role of the lymphoreticular system in pathogenesis may differ between natural scrapie and BSE infection in sheep of the ARQ/ARQ genotype (Jeffrey *et al.*, 2001). Other studies have shown that Suffolk, Lacaune and Texel sheep are also susceptible to experimental challenge with BSE (Schreuder & Somerville, 2003). However, to date no evidence of BSE in sheep under natural conditions has been observed.

Small ruminants faced the same risk factors to BSE infection as cattle, as they too were exposed to possibly infected meat and bone meal (MBM) albeit at much lower levels (Schreuder & Somerville, 2003). In addition, the likely transmission of BSE from cattle to humans causing vCJD indicated that BSE was capable of bypassing the species barrier and infecting a new host species. Previous studies have failed to replicate the likely natural routes of transmission of BSE to sheep. However, Foster, McKelvey, Fraser *et al.* (1999) showed that goats experimentally infected with BSE failed to transmit disease to recipient animals following embryo transfer. Furthermore, the offspring that developed from the embryos of the infected donor animals showed no evidence of disease. This study indicated that BSE was unable to transmit maternally in goats (Foster *et al.*, 1999). More recent studies involving greater animal numbers are currently under way in the UK and it is likely that these experiments will provide more data on the efficiency of the natural transmission of BSE to sheep (Foster *et al.*, 2001).

1.5 The prion protein

1.5.1 PrP^c biosynthesis

The mammalian PrP gene encodes for a protein termed PrP^c that is about 250 amino acids in length and contains several distinct domains. PrP^c is a sialoglycoprotein with a molecular weight of 33-35 kDa. In most species five copies of a proline/glycine-rich octapeptide N-terminal repeat are present, whilst bovine PrP^c contains between five and seven copies (Goldmann, Hunter, Martin *et al.*, 1991b, Schlapfer, Saitbekova, Gaillard *et al.*, 1999). In addition, a highly conserved hydrophobic motif and a C-terminal region, which is a signal for the addition of a glycosyl-phosphatidyl-inositol (GPI) anchor are present (Figure 1.2).

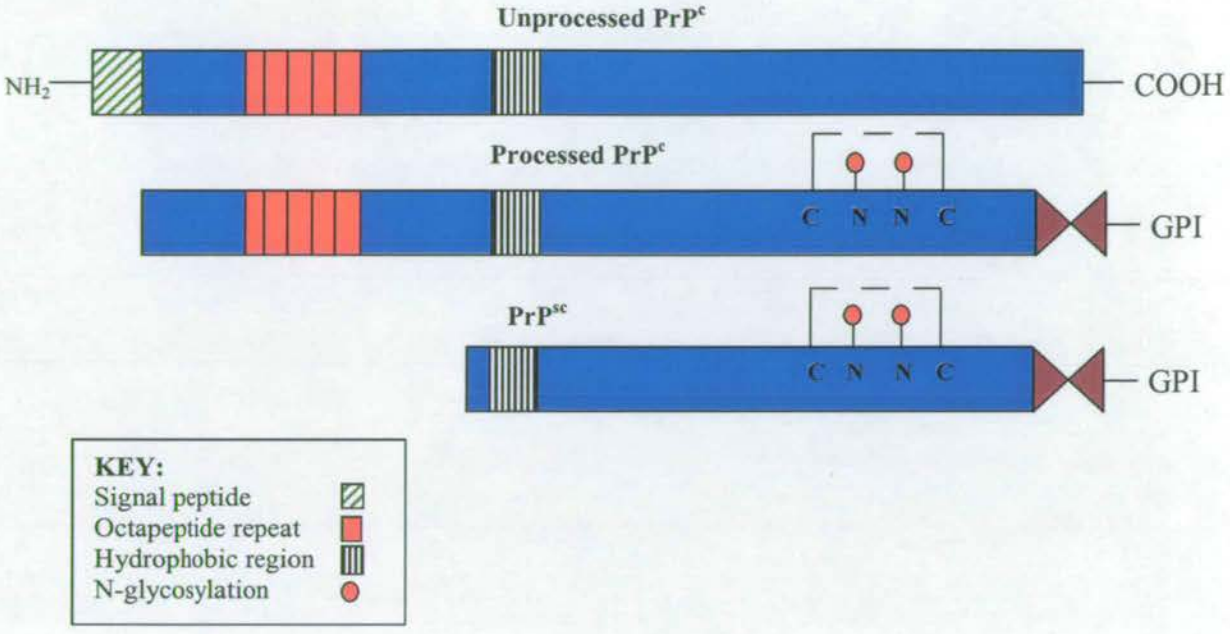


Figure 1.2 *PrP protein structures*

Unprocessed PrP^c is not fully glycosylated and contains an N-terminal signal peptide and a sequence at its C-terminus for the addition of a glycosyl-phospahtidyl-inositol (GPI) anchor. Processed PrP^c is cleaved at its N-terminal signal peptide, N-glycosylated at two arginine residues (N), a disulphide bridge is formed between two cysteine residues (179 & 214 (ovine numbering)) and a GPI anchor is added to its C-terminus.

As is consistent with other membrane proteins PrP^c is synthesised in the rough ER and passes through the golgi apparatus on its way to the cell surface (Hegde & Lingappa 1999). During its biosynthesis PrP^c is subjected to a number of post-translational modifications. The N-terminal signal peptide, which targets the protein to the rough endoplasmic reticulum (ER), is cleaved and N-linked oligosaccharide chains are added at two sites. A disulphide bond is formed between cysteine residues 179 and 214 (ovine numbering) and a GPI anchor is attached to the C-terminal end of the protein (Basler *et al.*, 1986, Hope, Morton, Farquhar *et al.*, 1986, Stahl, Baldwin, Burlingame *et al.*, 1990a, Stahl, Baldwin, Hecker *et al.*, 1992, Stahl, Borchelt, Hsiao *et al.*, 1987, Turk, Teplow, Hood *et al.*, 1988). Whilst in the ER the protein is processed and folded into its correct conformation following interactions with molecular chaperones. It is possible that these molecular interactions could have a role in the conversion of PrP^c to PrP^{sc} and this is supported

by the findings that PrP^c carrying pathogenic mutations showed altered biochemical properties within the ER (Daude, Lehmann & Harris, 1997).

Studies of PrP^c biosynthesis in cultured cells have shown that the protein undergoes two post-translational cleavages as part of its normal processing. The first occurs within the GPI anchor, releasing the protein from the cell membrane, and the second occurs within the conserved hydrophobic region (Harris, Huber, van Dijken *et al.*, 1993, Jimenez-Huete, Lievens, Vidal *et al.*, 1998). This second cleavage may take place in an endocytic compartment of the cell, or in a cholesterol rich domain of the plasma membrane (Harris, 1999, Taraboulos, Scott, Semenov *et al.*, 1995, Vey, Pilkuhn, Wille *et al.*, 1996). Cell studies analysing PrP^c biosynthesis showed that deletion of the C-terminal signal sequence resulted in the failure of the protein to attach itself to the cell membrane and as such it was secreted into the cell medium (Borchelt, Rogers, Stahl *et al.*, 1993, Rogers, Yehiely, Scott *et al.*, 1993). Cleavage of the GPI anchor by bacterial phosphatidyl-inositol-specific phospholipase C (PIPLC) had a similar effect, as PrP^c was cleaved from the membrane into the extracellular milieu (Borchelt *et al.*, 1993, Borchelt, Scott, Taraboulos *et al.*, 1990, Caughey, Neary, Buller *et al.*, 1990, Lehmann & Harris, 1995, Stahl *et al.*, 1990a).

Stahl, Borchelt & Prusiner (1990b) noted, that although PrP^c was released from the cell membrane by PIPLC, PrP^{sc}, was not cleaved by this enzyme. One explanation for this observation is that PrP^c and PrP^{sc} are differentially anchored to the cell membrane, thus restricting the action of the enzyme on PrP^{sc} (Stahl *et al.*, 1990b). It was initially thought that the C-terminal GPI anchor was important in the association of PrP^c with lipid-rich rafts, however recent studies by Walmsley, Zeng & Hooper (2003) showed that this association was still possible after the deletion, or substitution of the GPI anchor with a transmembrane domain. This association was only prevented when an N-terminal segment was deleted from the protein, indicating that the N-terminal region of the protein acts as a cellular raft-targeting signal (Walmsley *et al.*, 2003). Further studies by Nunziante, Gilch & Schatzl (2003) showed that deletion of the N-terminus of PrP^c inhibited the internalisation of the protein, resulting in a prolongation of its cellular half-life and a delay in its processing through the secretory pathway. PrP^c is synthesised and degraded very rapidly with a half-life of between 3-6 hours, whilst the two cleavage events occur

relatively slowly in comparison. It is therefore hypothesised that several different cleavage products of the protein may be present in the cell at any one time (Borchelt *et al.*, 1990, Caughey, 1993).

1.5.2 PrP^c glycosylation

Glycosylation is recognised as being an important aspect of most cell surface proteins and PrP^c contains two sites for N-glycosylation at Asn_{181}IleThr and Asn_{197}PheThr, both of which can be variably glycosylated (Oesch *et al.*, 1985). Differential glycosylation of these sites gives rise to three glycoforms of PrP^c, un-glycosylated, mono-glycosylated and di-glycosylated, and the ratio of these three glycoforms has been shown to differ between different brain regions (DeArmond, Qiu, Sanchez *et al.*, 1999, Rudd, Endo, Colominas *et al.*, 1999, Somerville, 1999, Vorberg & Priola, 2002). Within the ER the molecular weights of these PrP^c precursors are 25, 28 and 33 kDa for the un-, mono- and di-glycosylated forms, respectively. The N-linked oligosaccharide chains on PrP^c are further modified within the golgi apparatus, producing highly complex chains containing sialic acid, which are resistant to endoglycosidase H (Caughey, Race, Ernst *et al.*, 1989, Haraguchi, Fisher, Olofsson *et al.*, 1989). The molecular weights of these matured PrP molecules are 30, 33 and 35 kDa, respectively. Recent studies by Pan, Li, Wong *et al.* (2002) using two-dimensional immunoblots have shown that PrP^c glycosylation may be far more complex, as many different PrP^c species with differing molecular weights, levels of glycosylation and iso-electric points were observed. In addition, the N-glycan chains of both Syrian hamster and mouse PrP^c have been shown to differ, highlighting the complexity of the PrP glycosylation (Stahl, Baldwin, Teplow *et al.*, 1993, Stimson, Hope, Chong *et al.*, 1999)

It is generally accepted that N-glycans serve to stabilise the protein to which they are attached and interestingly studies have shown that the conversion of PrP^c to PrP^{sc} occurs more easily with un-glycosylated PrP^c (Imperiali & O'Connor, 1999, Taraboulos, Rogers, Borchelt *et al.*, 1990). Ma & Lindquist (1999) showed that PrP^{sc} and not PrP^c was expressed in immortalised murine neuroblastoma (N2a) cells cultured under reducing conditions, and in conditions shown to block N-glycosylation. These studies indicated that un-glycosylated PrP^c has a predisposition

to convert to PrP^{sc}, or to a PrP^{sc} like molecule and that the N-glycan chains may offer protection against this conversion (Winklhofer, Heske, Heller *et al.*, 2003). Furthermore, as the two N-glycan chains of PrP^{c} cover orthogonal faces of the protein it is hypothesised that they are capable of protecting large regions of the protein from the action of proteases and from non-specific protein-protein interactions by way of steric hindrance (Rudd, Wormald, Wing *et al.*, 2001).

1.5.3 PrP^{c} trafficking and sub-cellular location

PrP^{c} is clustered in detergent insoluble microdomains (DIMs), which contain cholesterol and glycosphingolipids (Taraboulos *et al.*, 1995). These DIMs are associated with signaling molecules, including, members of the Src family of tyrosine kinases and G protein subunits (Gorodinsky & Harris, 1995, Mahfoud, Garmy, Maresca *et al.*, 2002, Naslavsky, Stein, Yanai *et al.*, 1997a, Naslavsky, Stein, Yanai *et al.*, 1997b, Simons & Ikonen, 1997). Most of the information regarding PrP^{c} trafficking has been derived from PrP^{c} expression studies using transfected cell cultures. Following its biosynthesis PrP^{c} is trafficked to cell membrane DIMs, however it does not remain here and instead is internalised via clathrin-coated pits to an endocytic compartment (Gorodinsky & Harris, 1995, Shyng, Heuser & Harris, 1994, Shyng, Huber & Harris, 1993) (Figure 1.3). It should be noted that certain studies have suggested that the C-terminal signal sequence may help to target the protein to caveolae-like domains within the cell membrane (Harmey, Doyle, Brown *et al.*, 1995, Kaneko, Zulianello, Scott *et al.*, 1997, Peters, Mironov, Peretz *et al.*, 2003, Vey *et al.*, 1996). However, this is disputed by other researchers who have shown that these domains contain no caveolin (Gorodinsky & Harris, 1995).

Lehmann, Milhavet & Mange (1999) showed that PrP^{c} is endocytosed in cultured cells with a half-life of twenty minutes and is recycled back to the cell membrane with a transit time of one hour. Whilst in this endocytic compartment approximately 5% of the PrP^{c} molecules are cleaved at the second proteolytic cleavage site, which lies within the central hydrophobic domain of the protein (Harris, 2003). The remaining 95% of the internalised PrP^{c} molecules are recycled to the plasma membrane. In addition, some of this membrane bound PrP^{c} is released into the extracellular milieu following the cleavage of its GPI anchor (Hay, Prusiner

& Lingappa, 1987, Shyng *et al.*, 1993). The nature of this pathway suggests that it may function to promote the uptake of an as yet unidentified protein or molecule, possibly copper, either from the cell surface or from the extracellular milieu (Harris, 1999, Madore, Johnson-Kraines, Rothstein *et al.*, 1999). Yedidia, Horonchik, Tzaban *et al.* (2001) showed that approximately 10 % of PrP^c is targeted for degradation via the ER associated degradation (ERAD) proteasome pathway. During this pathway PrP^c is shifted into the cytosol where it is ubiquitinated and the authors suggested that the presence of ubiquitinated PrP^c within the cytosol could potentially have pathogenic implications (Yedidia *et al.*, 2001).

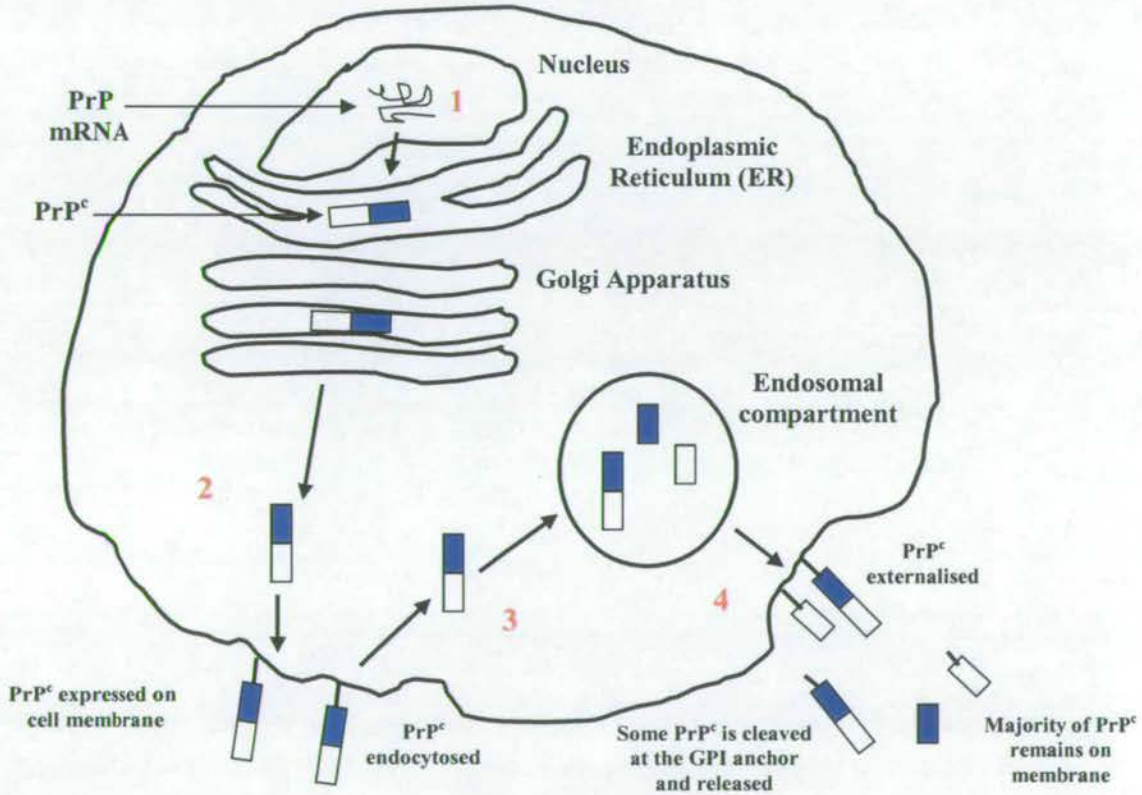


Figure 1.3 *PrP^c biosynthesis, trafficking and subcellular location*

1. PrP mRNA is translated into PrP^c in the rough endoplasmic reticulum and transported through the golgi apparatus to the cytoplasm. 2. PrP^c is trafficked to the cell membrane, where it is attached by its GPI (glycosyl-phosphatidyl-inositol) anchor. 3. PrP^c is then internalised into an endosomal compartment, where 5% of PrP^c is cleaved within the hydrophobic domain, the remainder of the internalised PrP^c is recycled back to the cell membrane. 4. Some of the PrP^c is cleaved at its GPI anchor and released from the cell membrane, but the majority remains on the cell membrane.

1.5.4 PrP^c topology

PrP^c is synthesised at the endoplasmic reticulum (ER), in three different topological isoforms (Hay *et al.*, 1987, Kim, Rahbar & Hegde, 2001, Lopez, Yost, Prusiner *et al.*, 1990, Stewart & Harris, 2003, Yost, Lopez, Prusiner *et al.*, 1990) (Figure 1.4). Secretory PrP^c ($^{Sec}PrP^c$) is fully translocated into the ER lumen and is attached to the lipid-bilayer by its C-terminal GPI anchor (Stewart & Harris, 2001). Two other forms of the protein have been isolated and these are termed $^{Ntm}PrP^c$ and $^{Ctm}PrP^c$. These two forms may span the lipid-bilayer once via the central hydrophobic region of the protein (residues Ala_{113}-Ser_{135}) (Stewart & Harris, 2001). The N-terminus of $^{Ntm}PrP^c$ lies within the ER lumen, whilst it is the C-terminus of $^{Ctm}PrP^c$ that lies within the ER lumen (Hegde, Voigt & Lingappa, 1998b, Kim *et al.*, 2001, Stewart & Harris, 2001). It is proposed that $^{Ntm}PrP^c$ lacks a GPI anchor, whilst $^{Ctm}PrP^c$ adopts an unusual mode of membrane attachment as it spans the lipid-bilayer via its hydrophobic region, and is also attached to the bilayer via its C-terminal GPI anchor (Kim *et al.*, 2001, Stahl *et al.*, 1987, Stewart & Harris, 2001).

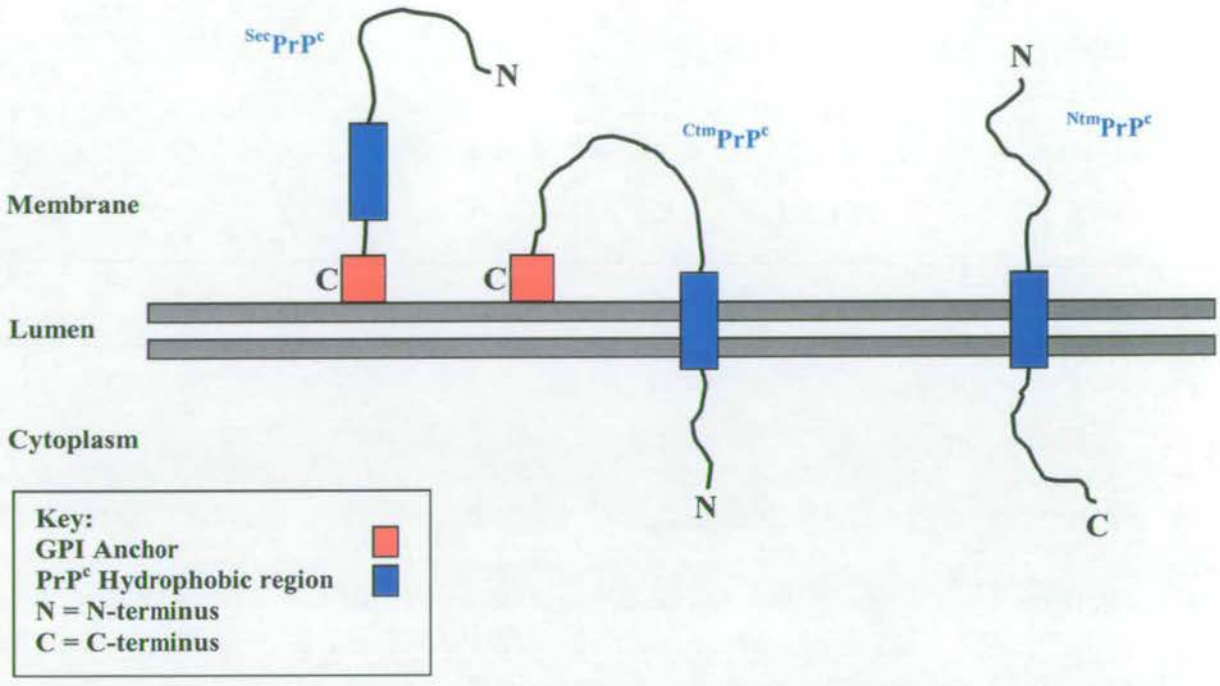


Figure 1.4 *Schematic of the three topological isoforms of PrP^c synthesized in the rough endoplasmic reticulum*

PrP^c is synthesized at the rough endoplasmic reticulum in three topological isoforms, $^{Sec}PrP^c$, $^{Ctm}PrP^c$ and $^{Ntm}PrP^c$. $^{Sec}PrP^c$ is attached to the bilayer via its C-terminus, $^{Ctm}PrP^c$ is attached via its C-terminus and also spans the bilayer via its hydrophobic region. $^{Ntm}PrP^c$ lacks a C-terminal GPI anchor and spans the bilayer via its hydrophobic region (Stahl *et al.*, 1987).

$^{Ctm}PrP^c$ has been implicated in prion disease pathogenesis, for example, transgenic PrP null mice expressing recombinant PrP^c molecules with mutations in or around the transmembrane domain, and within the stop transfer effector (STE) domain (Lys_{104}-Met_{112}), were shown to favour the $^{Ctm}PrP^c$ formation (Hegde *et al.*, 1998a, Hegde, Tremblay, Groth *et al.*, 1999, Stewart & Harris, 2003). In addition, mice expressing $^{Ctm}PrP^c$ developed a spontaneous neurological disease, but lacked detectable PrP^{sc} (Hegde *et al.*, 1998a, Hegde *et al.*, 1999). Therefore the authors suggest that it could be $^{Ctm}PrP^c$, and not PrP^{sc}, which is responsible for the neurodegeneration in familial and infectious prion diseases (Hegde *et al.*, 1998a, Hegde *et al.*, 1999). In addition, $^{Ctm}PrP^c$ was shown to accumulate in the brains of mice expressing a PrP transgene with an $Ala_{117}Val$ mutation, which is associated with GSS in humans (Hegde *et al.*, 1998a, Hegde *et al.*, 1999, Mastrianni, Curtis,

Oberholtzer *et al.*, 1995, Stewart, Drisaldi & Harris, 2001, Tranchant, Doh-ura, Warter *et al.*, 1992).

It is very likely that $^{Sec}PrP^{c}$ is the major species present on the cell membrane, whilst the amount of $^{Ctm}PrP^{c}$ in the cell is likely to be very low in comparison (Stewart & Harris, 2001, Stewart & Harris, 2003). However, it is not yet clear if $^{Ctm}PrP^{c}$ is a byproduct of PrP^{c} biosynthesis which may have a role in disease progression if overexpressed.

1.5.5 Structural characteristics of PrP^{c} and PrP^{sc}

PrP genes have been identified in a number of mammalian species and the PrP^{c} protein primary structure is highly conserved with an average of 90 % sequence homology (Schatzl, DaCosta, Taylor *et al.*, 1997). Ovine PrP^{c} comprises of 256 amino acids, human and hamster PrP^{c} 253 and mouse PrP^{c} 254 (Rivera-Milla, Stuermer & Malaga-Trillo, 2003, Schatzl *et al.*, 1997, van Rheede, Smolenaars, Madsen *et al.*, 2003). PrP^{c} has a high content of α-helices and is sensitive to proteolytic digestion, in contrast PrP^{sc} has a high content of β-sheet, and is partially resistant to proteolytic digestion in its aggregated form, it is also largely insoluble (Inouye & Kirschner, 1997, Meyer, McKinley, Bowman *et al.*, 1986, Nguyen, Inouye, Baldwin *et al.*, 1995, Oesch *et al.*, 1985, Pan, Baldwin, Nguyen *et al.*, 1993). PrP^{c} and PrP^{sc} do not differ in their primary structure, neither do they appear to differ in their post-translational modifications (Stahl *et al.*, 1993). PrP^{c} and PrP^{sc} share similarities in their SDS-PAGE mobility and in their antigenicity (Barry & Prusiner, 1986, Oesch *et al.*, 1985). In addition, the proteins are cleaved in a similar manner at their N-terminal peptide cleavage sites (Caughey, Race, Vogel *et al.*, 1988, Hope *et al.*, 1986, Oesch *et al.*, 1985, Stahl *et al.*, 1990a, Stahl *et al.*, 1990b). PrP^{c} and PrP^{sc} are glycosylated in a similar manner and a GPI anchor is attached to both proteins (Caughey *et al.*, 1988, Hope *et al.*, 1986, Oesch *et al.*, 1985, Stahl *et al.*, 1990a, Stahl *et al.*, 1990b). Therefore, the differing properties of the two PrP conformers must be reflected in their secondary or tertiary structure, but to date, it is still not clear how this takes place.

Aspects of the structure of PrP^{c} have been elucidated by nuclear magnetic resonance (NMR) studies in mice, Syrian hamster, cattle and humans (Calzolai,

Lysek, Guntert *et al.*, 2000, James, Liu, Ulyanov *et al.*, 1997, Riek, Hornemann, Wider *et al.*, 1996, Riek, Hornemann, Wider *et al.*, 1997, Riek, Wider, Billeter *et al.*, 1998, Zahn, Liu, Luhrs *et al.*, 2000). These results, along with other studies have shown that PrP^c is a monomeric protein, containing a globular domain stretching between residues 125-228, and an N-terminal flexible tail domain (Mehlhorn, Groth, Stockel *et al.*, 1996, Pergami, Jaffe & Safar, 1996). The PrP^c globular domain consists of a single double stranded anti-parallel β-sheet and three α-helices (Riek *et al.*, 1996). In addition, a number of species-specific variations have been identified in the structure of PrP^c (Riek *et al.*, 1998, Zahn *et al.*, 2000). Peretz, Williamson, Matsunaga *et al.* (1997) utilising recombinant antibody Fab fragments against linear epitopes on PrP^c and PrP^{sc}, showed that the major conformational changes between PrP^c and PrP^{sc} may exist in the N-terminal region (residues 90-120). They showed that epitopes in the C terminal region were accessible in both PrP^c and PrP^{sc}, but that epitopes in the N-terminal region were accessible in PrP^c but were buried or inaccessible in PrP^{sc} (Peretz *et al.*, 1997). Knaus, Morillas, Sweitnicki *et al.* (2001) produced a crystal structure of recombinant human PrP^c in a dimeric form, in addition, Meyer, Lustig, Oesch *et al.* (2000) showed that while PrP^c normally exists as a monomer, it may adopt a dimeric conformation.

1.5.6 PrP^c function

The normal function of PrP^c remains elusive although it is highly conserved throughout many species suggesting that it plays a key role in cellular metabolism. It has been suggested that due to the location of PrP^c on the cell surface it may be involved in synaptic function, cell to cell communication, cell adhesion, signal transduction or ligand uptake, amongst others (Brown & Besinger, 1998, Brown, Herms, Schmidt *et al.*, 1997, Herms, Madlung, Brown *et al.*, 1997, Mabbott, Brown & Bruce, 1997, Manson, McBride & Hope, 1992, Mouillet-Richard, Ermonval, Chebassier *et al.*, 2000). Experiments utilising the yeast two-hybrid system have shown that the laminin receptor protein (LRP) may act as a receptor for PrP^c (Rieger, Edenhofer, Lasmezas *et al.*, 1997, Rieger, Lasmezas & Weiss, 1999). Laminin and PrP^c both share the same binding site on LRP, which is a cell surface receptor that is highly conserved among mammalian species (Rieger *et al.*, 1997, Shmakov, Bode,

Kilshaw *et al.*, 2000). The expression of LRP on the cells of the intestinal brush border and in Paneth cell secretory granules is thought to be involved in both secretory and endocytotic functions (Rieger *et al.*, 1997, Shmakov *et al.*, 2000). More recent studies have shown that PrP^c binds to the laminin receptor in cultured cells and that this interaction may be important in mediating neuritogenesis (Gauczynski, Peyrin, Haik *et al.*, 2001, Graner, Mercadante, Zanata *et al.*, 2000, Hundt, Peyrin, Haik *et al.*, 2001).

PrP^c has been shown to selectively bind copper ions in a highly conserved region of its N-terminal domain termed the octapeptide repeat (PHGGGWGQ), thus pointing to a role in copper transport (Brockes, 1999, Brown *et al.*, 1997, Garnett & Viles, 2003, Prince & Gunson, 1998). Kramer, Kratzin, Schmidt *et al.* (2001) analysed copper binding to full length PrP^c, they showed that the N-terminal domain of PrP^c binds up to five copper ions and that this binding occurs within the physiological range. It has been shown that when PrP^c acquires copper during protein folding, it becomes endowned with superoxide dismutase (SOD) activity, and that this activity is abolished if the octapeptide repeat region is deleted (Brown & Besinger, 1998, Brown, Wong, Hafiz *et al.*, 1999, Rachidi, Vilette, Guiraud *et al.*, 2003). These experiments suggest that PrP^c function is related to its ability to bind copper in a pH dependent fashion and that it may be involved in the cellular response to oxidative stress (Brown, Clive & Haswell, 2001, Burns, Aronoff-Spencer, Legname *et al.*, 2003, Wong, Pan, Liu *et al.*, 2000). Interestingly, Vassallo & Herms (2003) suggested that PrP^c acts as a sensor for either copper ions, or free radical stimuli, triggering intracellular calcium signals which may finally result in the modulation of synaptic transmission or neuritogenesis. This hypothesis is supported by findings that calcium homeostasis and calcium and potassium currents are altered in PrP null mice (Herms, Korte, Gall *et al.*, 2000, Herms, Tings, Dunker *et al.*, 2001).

Tobler, Gaus, DeBoer *et al.* (1996) showed that PrP null mice, although otherwise normal, showed signs of increased brief awakening during sleep, altered circadian rhythms and electrophysiological abnormalities in synaptic function. More recently, Huber, DeBoer & Tobler (2002) showed that abnormalities in sleep patterns and circadian rhythms in PrP knockout mice are region-specific, indicating that regions of the brain, such as the frontal cortex, are unaffected by the loss of PrP^c

expression with regard to sleep regulation, while regions such as the occipital cortex are more seriously affected by this loss. The exact implications of these findings are not yet clear, but it is accepted that PrP^c may be involved in the function of circadian rhythms and that a loss of PrP^c expression acts to disturb brain activity (Huber *et al.*, 2002).

Mouillet-Richard *et al.* (2000) showed that in cultured cells PrP^c becomes coupled to tyrosine kinase fyn in a caveolin-1 dependent reaction, indicating that PrP^c may have a function in signal transduction. PrP^c has been shown to interact with several factors known to be involved in neuronal signaling *in vivo* (Spielhaupter & Schatzl, 2001). These findings have been added to more recently by studies which showed that PrP^c is involved in the transduction of neuroprotective signals through a cAMP/PKA-dependent pathway (Chiarini, Freitas, Zanata *et al.*, 2002).

It has been suggested that PrP^c may have a role as a nucleic acid chaperone protein (Derrington, Gabus, Leblanc *et al.*, 2002). Derrington *et al.* (2002) investigated this hypothesis by analysing the DNA chaperoning properties of PrP^c, they were able to show that PrP^c possesses all of the nucleic acid chaperoning properties of the retroviral nucleocapsid protein of human immuno-deficiency virus (HIV-1). The authors hypothesised that the function of PrP^c may be as a cellular defense mechanism against infection with enveloped viruses (Derrington *et al.*, 2002). This is supported by studies that showed that the expression of PrP mRNA in astrocytes is increased following infection with HIV-1 (Muller, Pfeifer, Forrest *et al.*, 1992, Muller, Rytik, Pfeifer *et al.*, 1990). More recent studies showed that the *in vitro* conversion of PrP^c to a PrP^{sc}-like molecule termed PrPres, was dependent on the presence of specific RNA molecules, indicating that RNA molecules may have a role in the pathogenesis of TSEs (Deleault, Lucassen & Supattapone, 2003).

1.5.7 *Prnd* (Doppel)

Moore, Lee, Silverman *et al.* (1999) established the DNA sequence downstream of the PrP gene in mice and discovered the open reading frame of a gene termed *Prnd*, located approximately 16 kb downstream of PrP, which encodes for a protein termed Doppel (dpl). Doppel genes have been discovered downstream of the PrP gene in humans, rats, sheep and cattle, therefore it is highly likely that other

mammalian species possess their own Doppel genes (Comincini, Foti, Tranulis *et al.*, 2001, Mead, Beck, Dickinson *et al.*, 2000, Moore *et al.*, 1999, Tranulis, Espenes, Comincini *et al.*, 2001). The *Prnd* gene encodes for a protein of 179 amino acids, which shows approximately 25% homology to the C-terminal part of PrP^c. As in PrP^c, Doppel is comprised of three α-helices, with a disulphide bond between α-helices two and three (Luhrs, Riek, Guntert *et al.*, 2003). In addition, Doppel lacks the octa-peptide repeat region located at the N-terminal of PrP^c.

Doppel messenger RNA (mRNA) is expressed at very high levels in testicular tissue, at slightly lower levels in other peripheral tissues, such as the heart and at very low levels in the brain (Moore *et al.*, 1999, Peoc'h, Serres, Frobert *et al.*, 2002, Silverman, Qin, Moore *et al.*, 2000, Tranulis *et al.*, 2001). Doppel is upregulated in the brains of the two PrP knock-out lines of transgenic mice, *Nagasaki* and *RcmO* due to an intergenic splicing event (Moore *et al.*, 1999, Moore & Melton, 1997, Sakaguchi, Katamine, Nishida *et al.*, 1996). Both of these lines showed signs of ataxia and apoptosis of cerebellar cells at around 70 weeks of age and the *Nagasaki* line showed loss of Purkinje cells (Moore *et al.*, 1999, Moore & Melton, 1997, Sakaguchi *et al.*, 1996). However, the two PrP knock-out lines *NPU* and *Zurich* I, do not over-express Doppel and remain healthy (Bueler *et al.*, 1992, Manson *et al.*, 1994a, Moore *et al.*, 1999, Moore & Melton, 1997, Nishida, Tremblay, Sugimoto *et al.*, 1999, Rossi, Cozzio, Flechsig *et al.*, 2001, Sakaguchi *et al.*, 1996).

More recently Tuzi, Gall, Melton *et al.* (2002) showed that the level of Doppel expression in the CNS of TSE infected mice had no effect on TSE disease incubation period or PrP^{sc} deposition. The authors concluded that Doppel had no detectable influence on TSE disease in the transgenic mice tested and therefore, Doppel is unlikely to be involved in naturally occurring TSEs (Tuzi *et al.*, 2002).

1.6 PrP genetics

1.6.1 PrP genetics in mice

Genetic analysis of the length of incubation period of the ME7 scrapie strain in mice led to the identification of *Sinc*, a single autosomal locus with two alleles, s7 and p7 (Dickinson & Mackay, 1964, Dickinson, Meikle & Fraser, 1968). It was

shown that these alleles control the incubation period of the ME7 strain, animals with the s7 allele have shortened incubation periods, whilst p7 animals have prolonged incubation periods (Dickinson & Mackay, 1964, Dickinson *et al.*, 1968). The mouse prion protein gene *Prnp* has been shown to control scrapie incubation time in mice. *Prnp* has two alleles $Prnp^a$ and $Prnp^b$ and two *Prnp* allotypes arise from codon 108 and 189 dimorphisms, PrP A and PrP B (Manson *et al.*, 1999, Westaway *et al.*, 1987). PrP A encodes 108 leucine (L)/189 threonine (T), whilst PrP B encodes 108 phenylalanine (F)/189 valine (V) (Manson *et al.*, 1999, Westaway *et al.*, 1987). These polymorphisms have been shown to have a major effect on the incubation period of scrapie in mice (Moore *et al.*, 1998). It is known that *Sinc* and *Prnp* are closely linked genes (Hunter, Hope, McConnell *et al.*, 1987), and a recent study by Moore *et al.* (1998) showed that these genes are congruent, indicating that in mice a single gene locus is responsible for the control of scrapie incubation period (Manson *et al.*, 1999). However, it is clear that polymorphisms of the PrP gene do not fully explain certain observations in experimental prion disease studies. For example, in mice, differences in the length of incubation period occur even when the amino acid sequence of the PrP genes present are identical (Carlson, Goodman, Lovett *et al.*, 1988, Dickinson, 1975, Kingsbury, Kasper, Stites *et al.*, 1983, Westaway *et al.*, 1987). Interestingly, it has been demonstrated that multiple quantitative trait loci are linked to the incubation period of prion disease in mice, however the exact nature of these loci remains to be investigated (Lloyd, Onwuazor, Beck *et al.*, 2001).

1.6.1.1 Transgenic studies in mice

Mice are not a natural host of scrapie, however they serve as a model system for transgenic studies and have added to our knowledge of the relationship between PrP^c expression and disease. For instance, PrP knock-out mice without a functional PrP gene appear to develop and behave normally (with the exception of abnormal circadian rhythms and disrupted sleep regulation) with a normal lifespan, thus eliminating the loss of PrP function as a primary cause of TSEs (Huber, Deboer & Tobler, 1999). Further experiments have shown that mice lacking PrP^c seem to be resistant to infection with scrapie infectivity, pointing to PrP^c having a major role in TSE pathogenesis (Bueler, Aguzzi, Sailer *et al.*, 1993, Weissmann *et al.*, 1994). It

has been shown that transgenic mice that over express PrP show shortened incubation periods (Race *et al.*, 1995, Raeber, Brandner, Klein *et al.*, 1998, Shmerling, Hegyi, Fischer *et al.*, 1998).

Manson *et al.* (1994b) used gene knockout technology to produce a mouse line (*NPU* line of 129/Ola mice) with an inactive PrP gene. Mice heterozygous for this mutation showed levels of PrP mRNA reduced by 50% when compared to the wild type mice. Using the ME7 strain of scrapie they infected wild type mice and mice heterozygous, and homozygous for the PrP null mutation. A gene dosage effect was observed whereby homozygote PrP null mice showed resistance to disease for up to 475 days, heterozygotes with one functioning PrP gene copy showed mean incubation periods of 220 days and wild type mice with two functioning PrP alleles showed shortened incubation periods with a mean of 130 days (Manson *et al.*, 1994b). The creation of transgenic mice which express mutated PrP gene sequences has shown that transgenic mice expressing PrP^{c} with N-terminal truncations develop a spontaneous neurological syndrome and present with ataxia and cerebellar lesions (Telling, 2000). In addition, a distinct spontaneous neurological phenotype was observed in mice with internal deletions in PrP^{c} (Raeber *et al.*, 1998).

Telling *et al.* (1995) produced two lines of transgenic mice, one expressed human PrP^{c} Tg(HuPrP) and the other line expressed a chimeric human/mouse PrP^{c} Tg(Mhu2M). In order to investigate the mechanisms underlying the transformation of PrP^{c} to PrP^{sc}, both of these lines of mice were inoculated with human brain extracts from inherited or sporadic prion disease cases. The Tg(HuPrP) mice showed resistance to disease, even though they were shown to express high levels of human PrP^{c}. However, their susceptibility to disease was promoted by the ablation of the mouse PrP gene, suggesting that in some way the endogenous mouse PrP^{c} inhibited the conversion of the human PrP^{c} into PrP^{sc}. Furthermore, the line of mice expressing chimeric PrP^{c} was found to be susceptible to disease and when the endogenous PrP gene was ablated only a moderate effect was observed on disease incubation times (Telling *et al.*, 1995). The authors hypothesise that a species-specific molecule, possibly a chaperone protein ('Protein X'), aids in the conversion of PrP^{c} to PrP^{sc} (Kaneko *et al.*, 1997, Telling *et al.*, 1995).

Crozet, Flamant, Bencsik *et al.* (2001) produced a line of PrP knockout transgenic mice expressing ovine PrP^c and these mice were inoculated intra-cerebrally with two natural scrapie isolates. Both isolates caused disease in the mice with mean incubation times of 238 and 290 days, whilst C57BL/6 mice, expressing only murine PrP^c, failed to develop disease, indicating that the species barrier had been bypassed by the expression of ovine PrP^c. In another study, Vilotte, Soulier, Essalmani *et al.* (2001) produced nine lines of PrP knockout transgenic mice expressing different ovine PrP^{VRQ} encoding transgenes. These mice were inoculated intra-cerebrally with two natural scrapie isolates derived from VRQ homozygous sheep. All of the inoculated mice developed disease and the incubation times were found to be inversely related to the steady state level of PrP in the brain (Vilotte *et al.*, 2001). Furthermore transgenic mice which over-expressed murine PrP^c were shown to be less susceptible to disease than the ovine PrP^c expressing mice, indicating that prion replication was favoured in the presence of ovine PrP^c (Vilotte *et al.*, 2001). These studies suggested that ovine PrP^c expressing transgenic mice lines may provide a viable model for the study of natural scrapie.

1.6.2 PrP genetics in sheep

In a similar way to the *Sinc* gene in mice, scrapie incubation periods in sheep are influenced by a gene termed *Sip*; which has two alleles, sA and pA (Hunter, Dann, Bennett *et al.*, 1992). The Neuropathogenesis Unit (NPU) South Country Cheviot flock contains two lines of sheep (positive and negative lines); each line is defined by the response to infection with the sheep scrapie brain pool number 1 (SSBP/1) isolate of scrapie (Dickinson *et al.*, 1968). The NPU flock of South Country Cheviots was founded in 1960 and has remained closed to the introduction of any new sheep since 1962 (Dickinson *et al.*, 1968). Sheep with the genotypes $Sip^{sA/sA}$ and $Sip^{sA/pA}$ are selected into the positive line (high susceptibility) and these succumb to subcutaneous challenge with SSBP/1, whilst those with the homozygous genotype $Sip^{pA/pA}$ fall into the negative line (low susceptibility) and do not succumb to subcutaneous challenge with SSBP/1 (Hunter, Foster, Dickinson *et al.*, 1989). The sA allele is at least partially dominant with SSBP/1, and $Sip^{pA/pA}$ offspring generated from $Sip^{sA/pA}$ heterozygote positive line sheep do not succumb to SSBP/1

subcutaneous challenge (Foster & Hunter, 1991, Hunter *et al.*, 1989). In contrast, the scrapie source CH1641 and BSE have been shown to have shortened incubation periods in a certain proportion of negative line sheep (Foster & Dickinson, 1988, Hunter, 1997). Using molecular genetics it has been shown that the *Sip* gene encodes the PrP protein and although there is a single PrP gene allele associated with Sip^{sA}, there are many PrP alleles associated with Sip^{pA} and sheep TSE genetics is now exclusively described in terms of the PrP gene.

1.6.2.1 Ovine PrP genotypes and polymorphisms

Many polymorphisms exist in the ovine PrP gene and three of these in particular have been associated with the incidence of both natural and experimental scrapie. Particular amino acid residues at three positions on the host PrP^c protein: codons 136, 154 and 171, are associated with disease susceptibility (Bossers, Schreuder, Muileman *et al.*, 1996, Goldmann, Hunter, Foster *et al.*, 1990, Hunter, Goldmann, Smith *et al.*, 1994a). Studies of the PrP protein encoding regions of various *Sip* alleles by Goldmann, Hunter, Benson *et al.* (1991a) identified PrP variants at codons 136, 154 and 171. Throughout this thesis, amino acid codon numbers are those of ovine PrP unless otherwise stated. The genotypes are described by giving the amino acid for each codon (136, 154 and 171) in turn, for each allele, in turn. Amino acids are described using the single letter code: A = alanine, H = histidine, R = arginine, Q = glutamine V = valine and X = any of the possible amino acids at that codon. Valine at codon 136 is encoded by sheep which are susceptible to experimental SSBP/1 infection, whilst sheep homozygous for alanine at codon 136 are resistant to peripheral SSBP/1 challenge. Therefore, NPU VRQ/VRQ homozygotes are 100% susceptible to subcutaneous infection with SSBP/1 scrapie and show short incubation periods of 167 days, whilst VRQ/ARQ and VRQ/ARR heterozygotes show more prolonged incubation periods of 250 days, and 322 days, respectively (Goldmann, Hunter, Smith *et al.*, 1994a).

PrP gene codon polymorphisms at position 171 show a disease linkage, with ARQ/ARQ homozygotes having shorter incubation periods following intracerebral inoculation with CH1641, whilst ARQ/ARR heterozygotes show lengthened incubation periods, and ARR/ARR homozygotes show resistance to infection

(Goldmann *et al.*, 1994a). Experimental challenge with BSE shows the same genotype linkage as CH1641 (Foster *et al.*, 2001, Goldmann *et al.*, 1994a, Goldmann, Hunter, Smith *et al.*, 1994b). In other flocks an additional polymorphism, histidine, exists for sheep at codon 171, but there is no evidence that it significantly influences the disease course (Hunter, 1998). However, different genotypes alone can not explain the varying responses of sheep to scrapie infection, as animals of different breeds with identical PrP^c protein allotypes show variations in their susceptibility to scrapie (Hunter, 1997). For example, Cheviot sheep in the NPU flock with the ARQ/ARQ genotype exhibit resistance to natural scrapie, whilst Suffolk sheep of the same genotype exhibit a high degree of susceptibility to natural scrapie infection (Hunter, 1997). It is therefore possible that other regions of the ovine PrP gene may contain disease-associated polymorphisms (Hunter *et al.*, 1994a).

1.6.3 PrP genetics in cattle

As only a small proportion of all infected cattle developed BSE disease, it was assumed that there is a similar genetic control of disease susceptibility in cattle as has been described for sheep. The bovine PrP gene is the prime candidate for the genetic control of disease susceptibility in cattle and there are three known allotypes in this gene (Goldmann *et al.*, 1991b). Different forms of the bovine PrP gene contain between five and seven copies of a G-C rich element within the protein coding exon (Goldmann *et al.*, 1991b, Schlapfer *et al.*, 1999). However, following the analysis of 370 cattle in Scotland no difference was observed between the frequencies of the different PrP genotypes in healthy and BSE infected animals (Hunter, Goldmann, Smith *et al.*, 1994b). Again, it is likely that other regions of the bovine PrP gene may contain disease-associated polymorphisms (Hunter *et al.*, 1994b).

1.6.4 PrP genetics in humans

The inherited forms of TSEs in humans i.e. GSS, fCJD & FFI, segregate with specific point mutations or insertions of octapeptide repeat regions in the coding

region of the human PrP gene (Prusiner, 1998). For example, Hsiao *et al.* (1989) showed that a mutation at codon 102 of the PrP gene which substituted proline for leucine is linked with the development of GSS. A number of other point mutations at codons 105, 117, 145, 198 & 217 and an insertion between codons 51 & 91 have been linked to GSS but are much rarer (Ghetti, Piccardo, Frangione *et al.*, 1996). A polymorphic change of methionine to valine at codon 129 appears to play a role in disease modulation (Goldfarb *et al.*, 1992). Individuals who are homozygous for methionine at codon 129 show an increased risk for developing sCJD, whilst individuals who are heterozygous (methioine/ valine) at the same codon exhibit a reduced risk of disease occurrence (Palmer, Dryden, Hughes *et al.*, 1991). In addition, FFI and fCJD were shown to segregate with different genotypes determined by a mutation at codon 178, which substituted asparagine for aspartic acid and the polymorphic change of methionine to valine at codon 129. FFI segregated with M at codon 129 and N at codon 178, whilst fCJD segregated with V at codon 129 and N at codon 178. Therefore, two distinct disease phenotypes appear to be linked to a single pathogenic mutation at codon 178 but are also determined by a common polymorphism at codon 129, when located on the same allele (Goldfarb *et al.*, 1992, Medori, Tritschler, LeBlanc *et al.*, 1992, Palmer *et al.*, 1991). However, some CJD cases are not associated with known mutations within the PrP gene open reading frame, therefore it is hypothesised that other mechanisms may control disease susceptibility in humans. For example, McCormack, Baybutt, Everington *et al.* (2002) identified polymorphic intron regions in the human PrP gene that were associated with cases of sporadic CJD (sCJD) and could therefore be involved in increased susceptibility to disease. The insertion of additional copies of the PrP gene octapeptide repeat region is associated with disease occurrence and patients with between 1-4 extra copies of the octapeptide repeat present with typical CJD and show no family history of neurological disorders. In contrast, patients with 5-9 extra copies of the octapeptide repeat show features of CJD, GSS or an atypical dementia, with an autosomal dominant pattern of inheritance (Rossi, Giaccone, Giampaolo *et al.*, 2000).

It is not clear how these mutations alter disease susceptibility, but it is suggested that inherited TSEs result from mutations in the PrP gene which cause

amino acid changes that may act to destabilise the tertiary structure of PrP^{c} (Cohen, Pan, Huang *et al.*, 1994, Harrison, Bamborough, Daggett *et al.*, 1997, Huang, Gabriel, Baldwin *et al.*, 1994). It is hypothesised that the mutated PrP^{c} present in these inherited TSEs has an increased propensity to convert to PrP^{sc} (Collinge, 1999).

1.7 PrP gene structure

1.7.1 PrP gene structure

The entire ovine PrP gene, derived from a Suffolk sheep, has previously been described (Lee, Westaway, Smit *et al.*, 1998) (Figure 1.5). The gene is conserved throughout different species; and comparisons of the open reading frames of non-human primates and humans revealed homologies of between 92.9-99.6% (Schatzl, Dacosta, Taylor *et al.*, 1995). In sheep, hamsters and mice exons I and II encode for the 5'untranslated region (UTR), whilst exon III contains a short 5' UTR sequence, the open reading frame and the entire 3'UTR (Lee *et al.*, 1998). There is no equivalent of exon II in the human PrP gene.

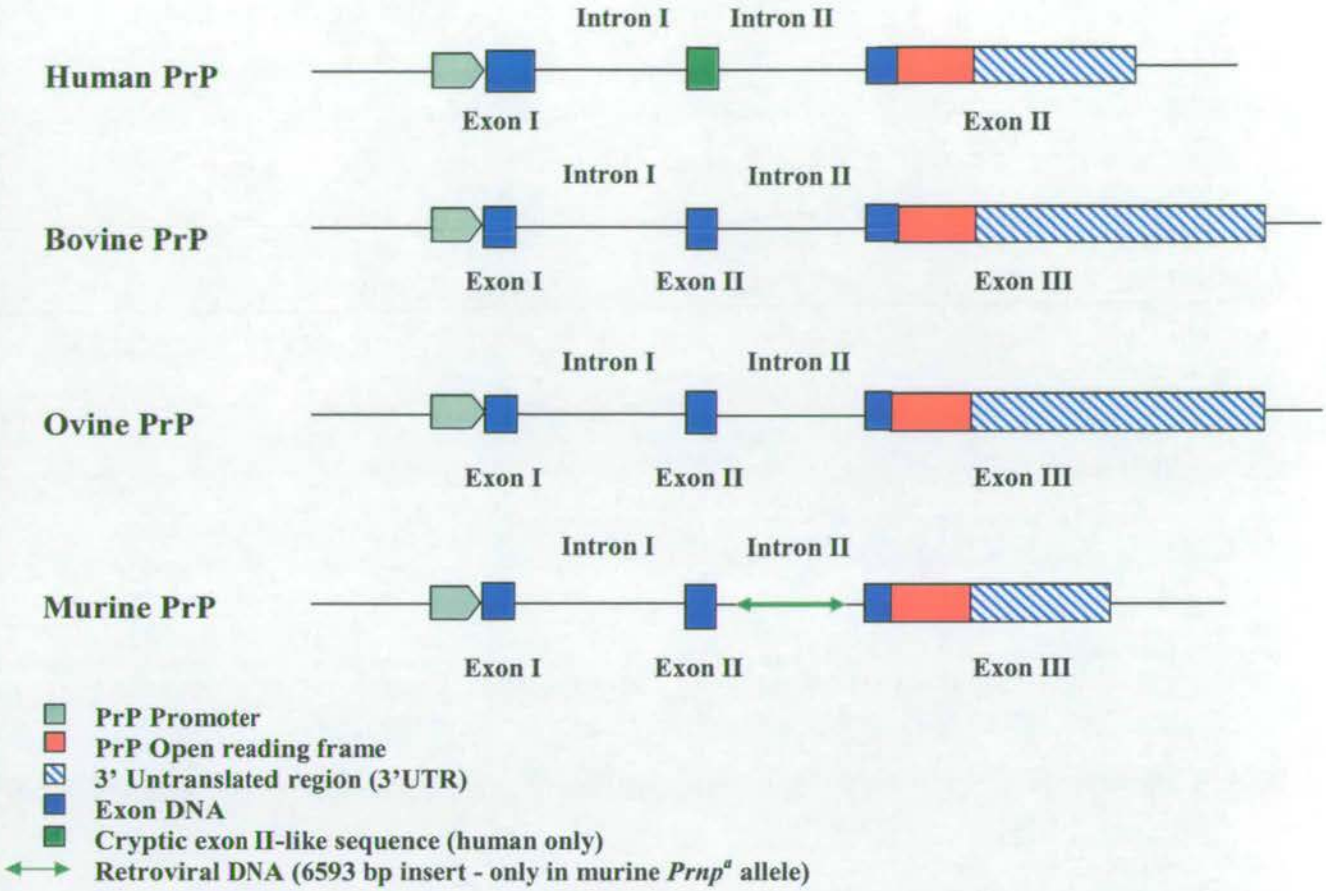


Figure 1.5 *Mammalian PrP gene organization*

Mammalian PrP gene structure consists of three exon sequences, exon III (exon II in humans) contains the open reading frame (ORF), a short 5' untranslated region (UTR) and the full 3'UTR. Human PrP gene exon I is ~130 bp in length, whilst exon I in the bovine, ovine and murine PrP genes is ~50 bp in length. Exon II has been experimentally defined in bovine, murine and ovine PrP but not in human PrP where a cryptic exon II-like sequence is found. Diagram is not accurately to scale. Human, murine & ovine PrP gene structures adapted from (Lee *et al.*, 1998), bovine adapted from (Hills, Comincini, Schlaepfer *et al.*, 2001).

Despite the variation in size of exon III throughout different mammalian species, the size of the protein-coding region of normal PrP genes remains relatively constant at approximately 760 bp. The location of the open reading frame remains constant, lying 10 bp from the intron/exon border, which is composed of a short 5'UTR sequence (Goldmann *et al.*, 1990).

1.7.2 PrP gene promoter region

In all mammalian species analysed so far the PrP gene promoter has been shown to lack typical regulatory elements associated with the initiation of transcription, such as a TATA box element (Funke-Kaiser, Theis, Behrouzi *et al.*, 2001, Inoue, Tanaka, Horiuchi *et al.*, 1997, Mahal, Asante, Antoniou *et al.*, 2001, Saeki, Matsumoto, Matsumoto *et al.*, 1996). However, the PrP gene promoter does possess a defined G-C rich region located proximal to the transcription start site (Baybutt & Manson, 1997, Westaway, Cooper, Turner *et al.*, 1994a, Westaway, Zuliani, Cooper *et al.*, 1994b). It is believed that these G-C rich elements act as potential binding motifs for zinc finger transcription factors such as specificity factor-1 (SP-1), and activator protein-2 (AP-2), which feature prominently in the regulation of genes which lack a TATA-box (Dynan, Saffer, Lee *et al.*, 1985, Dynan, Sazer, Tjian *et al.*, 1986, Dynan & Tjian, 1983, Mackay & Crossley, 1998, McKnight & Tjian, 1986, Rhodes & Klug, 1993, Struhl, 1989, Suske, 1999). As with other TATA-less gene promoters, a number of other transcription factor binding motifs are present in the PrP gene promoter, some of which appear to be species-specific (Baybutt & Manson, 1997, Funke-Kaiser *et al.*, 2001, O'Neill, Donnelly, Marshall *et al.*, 2003). In addition, the PrP promoter contains four motifs, which are conserved between all the mammalian species tested to date, forming candidate binding sites for, as yet unidentified transcription factors (Westaway *et al.*, 1994a, Westaway *et al.*, 1994b). O'Neill, Donnelly, Marshall *et al.* (2003) identified three polymorphisms in the ovine PrP gene promoter, one of which creates an SP-1 transcription factor binding motif in Cheviot sheep, which is otherwise absent in the ovine PrP gene promoter.

1.7.3 PrP gene introns

The ovine PrP gene contains two intronic sequences. The smaller intron I is approximately 2.4 kb in length, whilst intron II is some 14 kb in length (Goldmann *et al.*, 1990, Lee *et al.*, 1998). The bovine PrP gene introns are of the same size, however the introns of the other mammalian PrP genes do vary (Horiuchi, Ishiguro, Nagasawa *et al.*, 1998). Intron I of the mouse and hamster PrP genes is 2.2 kb and

intron II of the hamster PrP gene is 7.7 kb (Li & Bolton, 1997, Westaway *et al.*, 1994a). However, intron II in the mouse PrP gene varies between mouse strains, with an 18 kb intron II sequence being observed in mice with the *Prn-p*^{a} allele and a 12 kb sequence in mice carrying the *Prn-p*^{b} allele (Westaway *et al.*, 1994a). This difference is caused by the insertion of a 6.6 kb intracisternal A-particle (IAP) within intron II of the *Prn-p*^{a} allele (Kuff & Lueders, 1988, Lee *et al.*, 1998).

The human PrP gene contains one intron sequence of approximately 12.7 kb (Lee *et al.*, 1998). The role of the PrP gene intron regions in regulation of gene expression remains unclear, however, studies have revealed that the introns may influence the level of PrP gene expression in a tissue-specific manner. Baybutt & Manson (1997) showed that two independent sequences in the mouse PrP gene intron I were capable of repressing and activating PrP gene promoter activity. Fischer, Rulicke, Raeber *et al.* (1996) showed that by deleting intron II from a mouse PrP transgene expressed in PrP knockout mice it was possible to suppress the expression of PrP in the Purkinje cells of the cerebellum; in contrast, PrP gene expression in the other brain areas tested remained unaffected. These results indicated that in mice intron II contains sequences, which could be involved in the tissue-specific expression of the PrP gene. Inoue *et al.* (1997) characterised the bovine PrP gene promoter linked to a chloramphenicol acetyltransferase (CAT) reporter gene in cultured bovine fibroblast cells. They showed that the promoter was only able to function properly in conjunction with a section of intron I (+123 to +891). It is hypothesised that this section of intron I is involved in the promotion of the PrP gene and that this promotion may be carried out in a tissue specific manner (Inoue *et al.*, 1997). Finally, Lee *et al.* (1998) discovered the presence of three intron regions, which are conserved in the human, sheep and mouse PrP genes. One of these regions is located at the beginning of intron II and it is hypothesised that this region could be involved in the transcriptional regulation of the PrP gene in these species (Lee *et al.*, 1998).

1.7.4 PrP gene exons

The PrP gene contains three exons in all but the human PrP gene, which only has two exons. Exons I and II are both non-coding exons, whilst exon III (exon II in

humans) contains the full open reading frame and the complete 3'UTR (Lee *et al.*, 1998). The sequences of the PrP gene exons I and II are conserved between mammalian species, exon I is approximately 50 bp in size in all mammalian species, whilst exon II is approximately 100 bp in size in all except the human PrP gene. The human PrP gene lacks a defined exon II sequence but it does contain a cryptic exon II-like sequence (Lee *et al.*, 1998). The exon III sequence in ruminants is considerably longer than that observed in the other mammalian species and a variety of polymorphisms are observed in this region in different sheep breeds (Goldmann *et al.*, 1990, Lee *et al.*, 1998). The ruminant exon III sequence is approximately 4 kb in length, whilst in humans the equivalent exon II is 2.35 kb and in rodents exon II is 2 kb (Chesebro *et al.*, 1985, Goldmann *et al.*, 1990, Lee *et al.*, 1998, Puckett, Concannon, Casey *et al.*, 1991).

1.7.5 Ovine PrP gene 3' untranslated region (3'UTR)

Based on homology between PrP of different species the 3'UTR of the 4.6 kb PrP mRNA has been divided into regions A-G, with the 2.1 kb transcript containing only regions A-C (Goldmann *et al.*, 1990, Goldmann, O'Neill, Cheung *et al.*, 1999) (Figure 1.6). The human PrP gene 3'UTR shows sequence homology with regions A, B, C, E and G of the ovine 3'UTR, whilst the rodent 3'UTR only shows sequence homology to regions A, C, E and G (Goldmann *et al.*, 1990). The bovine PrP gene 3'UTR shows 85% homology to the ovine 3'UTR with the differences observed being spread throughout the 3'UTR (Horiuchi *et al.*, 1998). The disparity in size of exon III sequences between ruminants and non-ruminants is due to the presence of both long and short ruminant-specific interspersed repetitive sequences within the 3'UTR, which resemble transposable elements (Lee *et al.*, 1998). The largest of these ruminant-specific sequences displays homology to a mariner DNA transposon relic from the *Mellifera* (honeybee) subfamily of mariner elements and is present in all bovine and ovine 3'UTRs sequenced to date.

The 3'UTR of the ovine PrP gene has been shown to contain three potential sites for alternative polyadenylation, two of which are known to be functional (Goldmann *et al.*, 1990, Goldmann *et al.*, 1999). Sequence analysis has revealed the presence of a number of polymorphisms within the ovine 3'UTR, and has shown that

it contains several instability motifs (Goldmann *et al.*, 1999). The mRNA degradation in eukaryotic cells is a highly regulated process and may influence the level of gene expression, the presence of consensus instability motifs in the 3' UTR of the ovine PrP gene indicate that they may play a role in the degradation of PrP mRNA. These motifs are particularly prevalent in mRNAs encoding for proteins related to the inflammatory response (Caput, Beutler, Hartog *et al.*, 1986, Sachs, 1993). It is not clear if these sequences have an influence on the expression of the PrP gene and further studies are required to analysis any potential role.

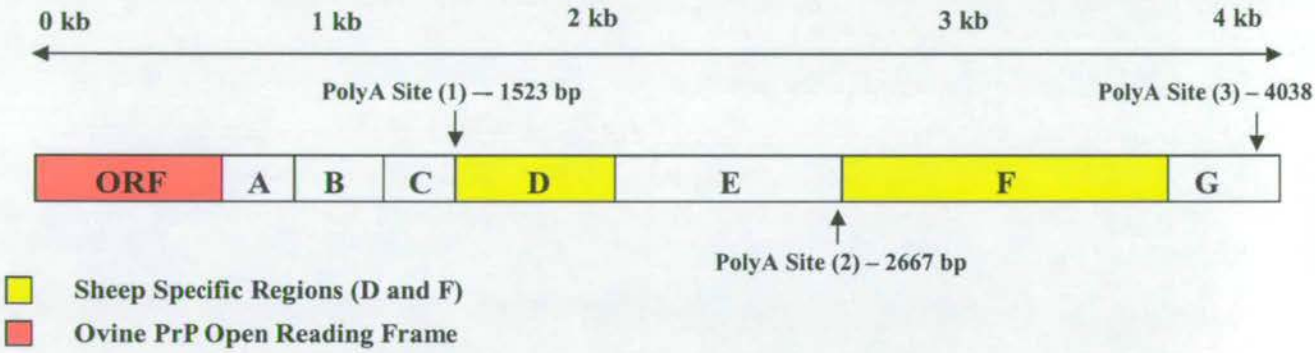


Figure 1.6 *Ovine PrP 3' untranslated region (3'UTR)*

Three alternative sites for polyadenylation are marked as PolyA Sites 1-3. The ovine PrP 3'UTR has been divided into seven distinct regions A-G based on sequence homology (Goldmann *et al.*, 1990). Regions D and F are sheep specific regions. Human PrP 3'UTR shows sequence homology with regions A, B, C, E and G, whilst the rodent 3'UTR shows sequence homology to regions A, C, E and G.

1.8 PrP gene expression

PrP^{c} expression is of critical importance to TSE development (Glatzel & Aguzzi, 2000). Manson *et al.* (1994b) showed that in mice both PrP^{sc} deposition and vacuolation are dependent upon PrP^{c} expression, and that this dependence is manifested in a dose dependent manner. To date, no significant difference has been reported in the level of PrP gene expression between normal and scrapie infected brain tissue. However, overexpression of the PrP gene in transgenic mice results in shortened incubation periods following inoculation with natural scrapie (Vilotte *et*

al., 2001). This study also showed that the incubation period appeared to be inversely related to the level of PrP^c expressed in the brain. Polymorphisms within the PrP open reading frame which contribute to disease susceptibility are well documented, however, they do not fully explain certain observations in disease transmission. It is therefore hypothesised that other regions of the PrP gene, such as the promoter region, or the structure of PrP mRNA are involved in scrapie susceptibility/ resistance. The exact mechanisms regulating PrP gene expression are not clear, however, unraveling these mechanisms is clearly of vital importance to our understanding of TSE pathogenesis.

1.8.1 PrP mRNA

In other eukaryotic genes the 3'UTR has been shown to carry a variety of regulatory motifs including sequences for mRNA processing, stability, transport and translocation and often contains several sequences for polyadenylation signals, which if present in the pre-mRNA can lead to the production of mRNAs with different sequences at the 3' end (Jackson & Standart, 1990, Mitchell & Tollervey, 2000). As mentioned above (Sections 1.8.4 & 1.8.5) the 3'UTR in ruminants is longer than that observed in other mammalian species and this seems to be due to the insertion of ruminant-specific sequences (Lee *et al.*, 1998). Two such regions of about 0.5 kb and 1.4 kb are linked with possible roles in ruminant-specific RNA processing and translation (Goldmann *et al.*, 1990, Goldmann *et al.*, 1999).

PrP mRNA of 4.6 kb is detectable in all tissues analysed and is at its highest in ovine brain tissue (Goldmann *et al.*, 1990, Horiuchi, Yamazaki, Ikeda *et al.*, 1995). In addition, a PrP mRNA of 2.1 kb is detectable by Northern blot in ovine peripheral tissue (Hunter, Manson, Charleson *et al.*, 1994c). The 2.1 kb mRNA is found in goats but has not been detected by this method in bovine tissue and is very rarely observed, if at all, in ovine brain (Goldmann *et al.*, 1999, Horiuchi *et al.*, 1995). There is no equivalent of the short 2.1 kb mRNA in rodents or humans. The 4.6 kb mRNA arises from a polyadenylation signal at position 4063, 25 bp downstream of the polyadenylation sequence at position 4038 (Polyadenylation site 3, Figure 1.6). The 2.1 kb mRNA arises from an alternative polyadenylation signal at position 1546, 23 bp downstream of the polyadenylation sequence at position 1523

(Polyadenylation site 1, Figure 1.6). In addition, a 3.3 kb mRNA transcript was detectable in ovine kidney tissue and this mRNA species made up between 1-5 % of the total PrP mRNA in this tissue (Polyadenylation site 2, Figure 1.6) (Goldmann *et al.*, 1999). Goldmann *et al.* (1999) isolated mRNAs from sheep lymph node and showed that the 5'UTRs of both the 4.6 kb and 2.1 kb transcripts are identical. However, as would be expected the shorter transcript is lacking several motifs present in the 3'UTR of the longer transcript i.e. instability motifs, transposable elements, repetitive sequences, although, both transcripts encoded for the same protein coding ORF (Goldmann *et al.*, 1999, Hunter *et al.*, 1989). The fact that both transcripts are observed in different tissues at varying levels leads to the possibility that alternative polyadenylation may be responsible for tissue-specific expression of PrP.

1.8.2 Developmental expression of PrP^c

Prenatal PrP^c expression can be detected in rat and hamster brain tissue immediately prior to birth. McKinley, Hay, Lingappa *et al.* (1987) showed that PrP mRNA expression was undetectable until 1 day after birth in hamsters and that it remained at a very low level of expression until 10 days after birth, peaking between 10 and 20 days of age, which was continued throughout adulthood. Lieberberg (1987) showed that PrP mRNA levels increased in a transcriptionally dependent manner during normal development. Manson, McBride & Hope (1992) detected PrP mRNA by *in situ* hybridisation in the developing mouse brain and spinal cord during embryonic development. PrP mRNA was detectable at 13.5 days and was also detected in the peripheral nervous system, ganglia, nerve trunks of the sympathetic nervous system and in neural cell populations of sensory organs (Manson *et al.*, 1992). Peripheral expression of PrP mRNA was observed in specific cell populations of 13.5 and 16.5 day-old embryos, and was also found to be present in extra embryonic tissues after 6.5 days (Manson *et al.*, 1992).

Moser, Colello, Pott *et al.* (1995) showed that the level of glial cell PrP mRNA expression in neonatal animals was comparable to that observed in neurones and that it increased by two-fold during postnatal development. Miele, Alejo Blanco, Baybutt *et al.* (2003) showed that in the developing mouse brain PrP mRNA

expression and PrP^c levels both increased during postnatal development. PrP mRNA expression is detectable throughout all stages of development in sheep, and expression of both the 2.1 kb and 4.6 kb ovine PrP mRNA species has been demonstrated in the foetus, lamb and adult sheep brain (Goldmann *et al.*, 1999). PrP mRNA is detectable from day 98 of gestation and increases by 100 fold at day 134 and doubles in lambs, this level of expression is then maintained throughout adulthood (Goldmann *et al.*, 1999). The developmental levels of the 2.1 kb and the 4.6 kb mRNA species vary depending on the type of tissue tested. The expression of the 2.1 kb species was found to be at its highest levels in spleen and kidney and at its lowest in brain tissue (Goldmann *et al.*, 1999).

1.8.3 Tissue-specific expression of the PrP gene

PrP gene expression, as measured by Northern blot analysis, is generally at its highest in the brain, intermediate levels of expression are detected in peripheral tissues, such as the heart and lungs, whilst the lowest levels are found in the liver where PrP expression is almost undetectable (Brown, Goller, Rudelli *et al.*, 1990, Caughey *et al.*, 1988, Goldmann *et al.*, 1999, McLennan, Rennison, Bell *et al.*, 2001, Oesch *et al.*, 1985). The PrP^c protein is widely expressed in a large variety of tissue types, it is abundant in neurones, and has been detected in both the neuronal cell body and in the axon (Ford, Burton, Morris *et al.*, 2002). PrP^c has been detected in the spleen, lymphoid tissues and lymphoid cells, such as follicular dendritic cells, lung, heart, kidney, skin, skeletal muscle, uterus, testes, blood vessels and red blood cells, adrenal gland, parotid gland, intestine, pancreas, proventriculus, abomasum and mammary gland (Barclay, Houston, Halliday *et al.*, 2002, Brown, Stewart, Ritchie *et al.*, 2000, Ford *et al.*, 2002, Horiuchi *et al.*, 1995, Vostal, Holada & Simak, 2001).

Horiuchi *et al.* (1995) observed that PrP mRNA levels in sheep were five times higher in brain than in kidney, whilst the amount of PrP^c protein detected in the brain was forty times higher than that seen in the kidney. It is therefore hypothesised that the levels of translational efficiency, protein synthesis and protein degradation differ between these different tissue types. In addition, Ford *et al.* (2002) showed that in mice there is a distinct lack of correlation between the expression of PrP^c and the expression of PrP mRNA in neurones, indicating that the steady state level of PrP^c

may be controlled in a post-transcriptional manner and may therefore be regulated by protein degradation or differential protein trafficking. PrP gene expression can differ within the same tissue; certain areas of the brain show very low, or no PrP mRNA expression i.e. caudate nucleus, neocortex and the granular layer of the hippocampus (DeArmond, Kristensson & Bowler, 1992, Kretzschmar, Prusiner, Stowring *et al.*, 1986). In contrast, other areas of the brain in hamsters show much higher levels of PrP gene expression, such as the cortex, striatum, cerebella Purkinje cells and neurones of the septum, thalamus and the caudate putamen (DeArmond, Mobley, DeMott *et al.*, 1987, Manson *et al.*, 1992, Sales, Rodolfo, Hassig *et al.*, 1998). Finally, Liu, Zwingman, Lee *et al.* (2001) demonstrated differential expression of PrP^c throughout different areas of the brain in mice using a panel of anti-PrP monoclonal antibodies. Interestingly they found no detectable PrP^c in the cerebellar Purkinje cells.

Collectively these studies indicated that PrP gene expression is subject to regulation in both a tissue and cell type-specific manner and it is hypothesised that this regulation is controlled by specific sequences within the non-coding regions of the PrP gene such as the promoter and the 3'UTR.

1.8.4 Factors that influence PrP expression

Wion, Lebert & Brachet (1988) showed that PrP mRNA levels are affected by nerve growth factor (NGF) which acted to increase PrP mRNA in PC12 cells. The highest levels of PrP mRNA were observed 7 days post treatment, however, a significant increase was detected at 48 hours post treatment. Lazarini, Castelnau, Chermann *et al.* (1994) demonstrated that NGF increases levels of PrP mRNA in cultured PC12 cells, a four-fold increase was observed after seven days of treatment and the same increase was observed following treatment with the cytokine interleukin-6 (IL-6). Kuwahara, Kubosaki, Nishimura *et al.* (2000) showed enhanced expression of PrP^c by NGF in immortalized mouse neuronal precursor cell cultures. Furthermore, Mobley, Neve, Prusiner *et al.* (1988) were able to show that PrP mRNA levels were induced by NGF in the developing hamster brain.

Lasmezas, Deslys & Dormont (1993) showed that recombinant human growth hormone (hGH) treatment of cells resulted in a 30 % increase in PrP mRNA

levels by 7 days post-treatment and the treatment of cells with insulin-like growth factor I (IGF-I) resulted in a dose dependent increase in PrP mRNA levels. In addition, Kuwahara *et al.* (2000) demonstrated that insulin acts to enhance PrP gene expression. Sauer, Wefer, Vetrugno *et al.* (2003) observed that PrP^c expression was increased following treatment of cells with ATP, NGF, epidermal growth factor (EGF) and tumor necrosis factor-alpha (TNF-alpha). Furthermore, Satoh, Kurohara, Yukitake *et al.* (1998) tested the effect of a panel of cytokines on PrP gene expression in human neuronal cell cultures, increases in expression of between 2.7-4.2 fold were observed following treatment with IL-1β, TNF-α, and phorbol 12-myristate 13-acetate, whilst a 50 % decrease in expression was observed following treatment with interferon-gamma (IFN-γ). Finally, Rybner, Hillion, Sahraoui *et al.* (2002) showed that all-trans retinoic acid acted to down regulate PrP mRNA levels in cultured cells, the authors hypothesised that this factor may be useful as a preventative strategy to slow down the development of TSE disease. These results show that the expression of the PrP gene is constitutive in a wide variety of human neuronal cell cultures and is controlled by a wide selection of cytokines, hormones and other growth factors.

1.9 Post transcriptional regulation of PrP mRNA

Studies in murine N2a cells, employing reporter gene constructs, showed that levels of expressed protein were affected by the length of the 3'UTR (Goldmann *et al.*, 1999). The highest protein levels were associated with the shortest 3'UTR, whilst the lowest levels were associated with the full length 3'UTR (regions A-G) (Goldmann *et al.*, 1999). Regions D-F contain almost 2 kb of ruminant-specific transposable elements (Lee *et al.*, 1998), which are present in the 4.6 kb transcript, but are missing in the 2.1 kb transcript. It is therefore possible that sequences within this region may interact with sheep-specific factors to regulate PrP gene expression (Goldmann *et al.*, 1999).

Further work carried out at the NPU by Marshall (2000) involved developing transient transfection methods for the introduction of reporter gene constructs into various ovine cell cultures including; Immortalised Cheviot cell cultures, sA80BR

(susceptible brain cell culture of genotype VRQ/VRQ) and pA80BR (resistant brain cell culture of genotype ARQ/ARR); Primary Icelandic cell cultures (of genotype ARQ/ARQ), IS120Cer (cerebellum derived cell culture) and IS120Liv (liver derived cell culture) (Sections 2.5.1-2.5.3). This work resulted in the identification of a region between nucleotides 2000-2700 that showed a tendency to reduce PrP^c expression levels in ovine brain cells derived from Cheviot sheep (Marshall, 2000). It was hypothesised that this region could contain a sheep-specific binding site(s) for an inhibitory binding protein(s) that could regulate gene expression by interfering with the transcription of the PrP gene, or by affecting the stability of the PrP mRNA transcripts (Marshall, 2000). Finally, Schroder, Nickodemus, Jurgens *et al.* (2002) showed that upstream AUG sequences in mouse PrP mRNA may act to regulate its translational efficiency *in vitro* in murine N2a cells.

1.9.1 mRNA processing in eukaryotes

Messenger RNA (mRNA) acts as a template for the synthesis of protein and is produced by the transcription of a particular gene by RNA polymerase II within the nucleus (Wahle & Keller, 1996). The initial mRNA transcript is termed the pre-mRNA and contains the non-coding intron sequences, which are removed by splicing (Staley & Guthrie, 1998). Splicing can be regulated by the availability of more than one splice site (alternative splicing) and the selection of one site over another can be further regulated by the action of hormones (Webster & Huang, 1999). Furthermore, splicing can be controlled in a tissue-specific manner by limiting the availability of specific splicing factors to certain tissue types (Venables & Eperon, 1999).

The 5' end of the mRNA is then capped by the addition of a modified nucleotide termed, 7-methylguanosine to the first nucleotide of the mRNA (Varani, 1997). Capping acts to protect the mRNA from degradation by 5' exonucleases in the cytoplasm and it is also a signal, which aids in the recognition of the start of the mRNA by the ribosome (Varani, 1997). The 3'end of the mRNA is then modified by polyadenylation, which is the addition of a poly A tail to the mRNA (Wahle & Keller, 1996). Polyadenylation involves the cleavage of the mRNA approximately 20 bp downstream of the polyadenylation site by a group of proteins termed the cleavage and polyadenylation specificity factor (CPSF) which bind to the signal

sequence AAUAAA (Bienroth, Keller & Wahle, 1993, Bienroth, Wahle, Suter-Crazzolara *et al.*, 1991, Gilmartin & Nevins, 1989, Murthy & Manley, 1992). Poly A polymerase then adds a string of adenine residues to the 3' tail of the mRNA, which act to prevent degradation by 3' exonucleases (Wahle & Keller, 1996). The number of adenine residues added can vary greatly but is generally between 20-200 nucleotides (Wahle & Keller, 1996).

1.9.2 Control of gene expression by alternative polyadenylation

In many eukaryotes a particular pre-mRNA species can give rise to more than one type of mRNA as a result of alternative polyadenylation. The choice of which sequence to use may be asserted in a tissue-specific manner. Therefore, mature mRNAs containing identical coding regions, but with differing stabilities or locations are capable of being produced by the same gene. For example, alternative polyadenylation of a cAMP-responsive element modulator-tau (CREM-τ) pre-mRNA is controlled by follicle stimulating hormone (FSH) (Foulkes, Schlotter, Pevet *et al.*, 1993). This results in the use of an upstream polyadenylation site, which excludes an instability element from the final mRNA transcript and results in an increase in CREM-τ levels (Foulkes *et al.*, 1993).

1.9.3 Alternative polyadenylation of ovine PrP mRNA

As mentioned previously two sheep PrP mRNAs of varying size (a result of alternative polyadenylation) have been identified (4.6 kb and 2.1 kb) in different tissue types in sheep. In addition, an intermediate sized mRNA transcript may exist (Goldmann *et al.*, 1999). Alternative polyadenylation is used to alter the translational efficiency of the β-amyloid precursor protein (APP), where two mRNA transcripts are observed, 3.2 and 3.4 kb (DeSauvage, Kruys, Marinx *et al.*, 1992). The longer mRNA transcript produces more protein than the shorter mRNA species as tested in *Xenopus* oocytes. The authors concluded that, as it is generally accepted that long mRNA transcripts are no more stable than short mRNA transcripts, the sequence between the two polyadenylation sites of the APP mRNA must act to increase mRNA translation (DeSauvage *et al.*, 1992). However, the use of alternative

polyadenylation sites does not explain why a PrP construct with a full length 3'UTR is expressed at lower levels than PrP constructs with shorter 3'UTRs (Goldmann *et al.*, 1999)? Inhibitory regions within the 3'UTR may have been deleted in the shorter constructs, but remain present in the longer constructs, thus explaining the anomalies in protein expression.

1.10 Transcriptional regulation of PrP gene expression

Each cell in the body contains identical genetic information, and the wide variety of cell types observed arises from the differential expression of this information, producing different combinations of proteins at any time (Tjian, 1995, Tjian & Maniatis, 1994). Gene expression is regulated by specific sequence elements within the promoter region which form specific binding motifs for transcription factors (Mitchell & Tjian, 1989). Transcription factors either aid in the formation of the basal transcription complex, in conjunction with RNA polymerase II, or they act to regulate the level of transcription of a particular gene through specific interactions with other transcriptional elements (Mitchell & Tjian, 1989, Tjian, 1995). Transcription is the first stage in gene expression and involves the synthesis of RNA from a DNA template, which is catalysed by RNA polymerase II (Buratowski, 1994, Chalut, Moncollin & Egly, 1994). Gene transcription can be conveniently divided into three stages, initiation, elongation and termination, and each of these stages are described below.

1.10.1 Transcriptional initiation in eukaryotes

Promoter sequences commonly contain a TATA box sequence approximately 25 bp upstream of the transcription start site (consensus site TATA(A/T)A(A/T)); and RNA polymerase II binds specifically to this sequence in conjunction with a number of general transcription factors (GTFs) (Chalut *et al.*, 1994, Orphanides, Lagrange & Reinberg, 1996). The TATA-binding protein (TBP) is a GTF, which binds to DNA at the TATA box, unwinding the DNA (Burley, 1996, Hampsey, 1998) (Figure 1.7). TBP binding to the TATA box is aided by various TAF_{II}s in a complex known as TFIID (Burley & Roeder, 1996, Conaway & Conaway, 1993,

Green, 2000, Ptashne, 1988, Verrijzer & Tjian, 1996). The binding of TFIID to the DNA is further enhanced by another GTF termed TFIIA, which acts to prevent the binding of inhibitory factors (Roeder, 1996). In addition, transcription factors like SP-1 are able to activate transcription through interactions with $TAF_{II}s$ (Hilton & Wang, 2003, Hoey, Weinzierl, Gill *et al.*, 1993). Another GTF termed TFIIB is able to bind to this multi-protein complex and consequently enables RNA polymerase II to bind (Figure 1.7). A whole series of additional GTFs (TFIIE, TFIIF, & TFIIH) can subsequently bind, forming the basal RNA polymerase II transcription initiation complex (Goodrich & Tjian, 1994, Roeder, 1996) (Figure 1.7).

Genes that lack a TATA box, such as PrP and APP, usually have an initiator (Inr) element, which overlaps the transcription start site. The transcription of genes whose promoter lacks a TATA box is often mediated by a combination of factors that bind to SP-1 and AP-2 motifs, and Inr elements (Hilton & Wang, 2003, Smale, 1997, Zhou & Chiang, 2001). In a similar way to the TATA box, the Inr element acts as a binding site for the GTF complex TFIID (Conaway & Conaway, 1993, Verrijzer & Tjian, 1996). As in TATA box containing promoters, once TFIID is bound it aids in the binding of other members of the basal transcription complex, allowing RNA polymerase II to bind to the promoter, and initiating transcription (Goodrich & Tjian, 1994, Roeder, 1996) (Figure 1.7). Therefore, the TATA box and the Inr element are functionally analogous to each other, as they both accurately direct transcription (Burley, 1996, Smale, 1997).

The expression of genes whose promoter region lacks a TATA box can be further regulated by the presence of a downstream promoter element (DPE), located at approximately 30 bases downstream of the transcriptional start site / Inr element (Burke & Kadonaga, 1996). The DPE is a 7 bp sequence that is involved, along with the Inr element, in the binding of the GTF complex TFIID (but not TBP) to the promoter (Burke & Kadonaga, 1997). In this way the cell is able to initiate transcription from genes which lack a TATA-box by utilising the Inr element and DPEs in union (Burke & Kadonaga, 1997).

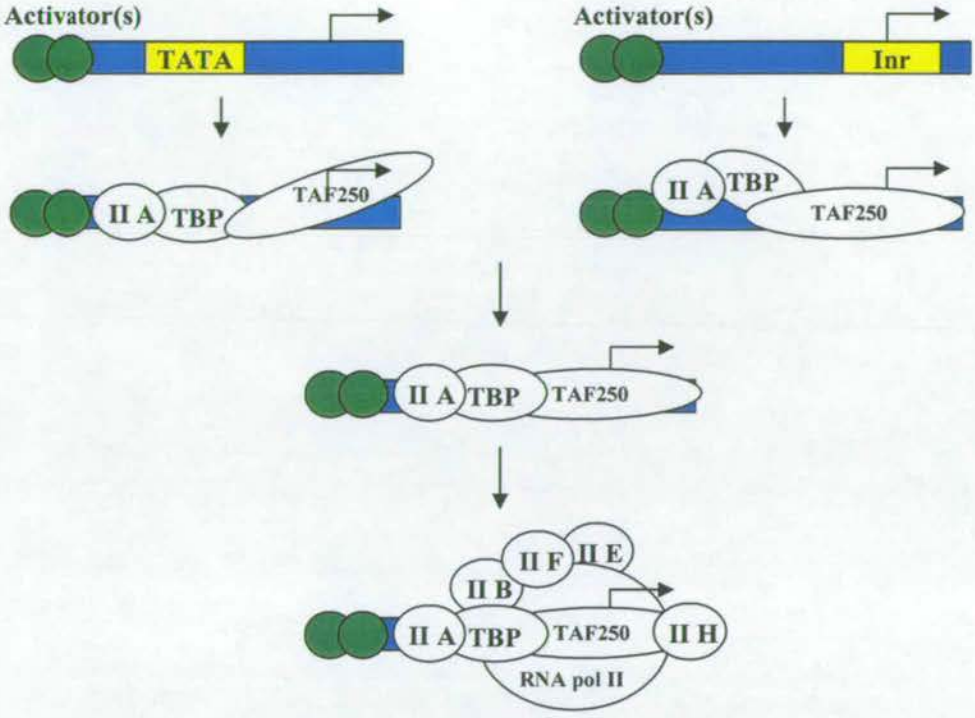


Figure 1.7 *Transcriptional initiation from a eukaryotic promoter, with & without TATA box*

Gene transcription from a promoter that contains a TATA box is performed in conjunction with TATA box binding protein (TBP), IIA & TAF250 and involves a direct interaction between TBP and the TATA box DNA sequence. Gene transcription from a TATA-less promoter involves the same factors but does not require a direct interaction between TBP and the DNA. RNA polymerase II (RNA pol II) is then able to bind in conjunction with a series of general transcription factors (IIB, IIE, IIF, IIH) to initiate gene transcription.

Transcriptional initiation of genes whose promoters lack a TATA-box is often associated with the regulatory transcription factors SP-1 & AP-2 (Kadonaga, Carner, Masiarz *et al.*, 1987, Philipsen & Suske, 1999, Salbaum, Weidemann, Masters *et al.*, 1989). For example, SP-1 has been shown to interact specifically with a GTF termed $TAF_{II}110$ (Hilton & Wang, 2003, Hoey *et al.*, 1993, Laity, Lee & Wright, 2001). It is thought that regulatory transcription factors act to alter the rate of transcription of specific genes by interacting with the basal transcription complex. It is likely that the binding of regulatory transcription factors represents a major method of controlling the rate of transcriptional initiation from TATA-less promoters

and is likely to be involved in the transcription of the PrP gene (Mitchell & Tjian, 1989). Additional promoter elements such as the CCAAT box (CAT box) can act as binding motifs for other transcription factors that may influence the rate of transcription initiation, however the PrP gene promoter contains no obvious CAT boxes (Tjian, 1995). Furthermore, upstream elements may act to influence gene transcription, such as enhancers, which can greatly stimulate transcription, and silencers which act to inhibit transcription (Atchison, 1988, Tjian, 1995). Finally, in the bovine PrP gene, sequences within intron I may act to regulate PrP gene expression, by interacting with the promoter region via an as yet undefined mechanism (Inoue *et al.*, 1997). A similar situation may exist in humans, as polymorphic intron regions in the human PrP gene are associated with cases of sporadic CJD (sCJD) (McCormack *et al.*, 2002).

1.10.2 Transcriptional elongation and termination in eukaryotes

Following transcriptional initiation, RNA polymerase II proceeds to produce the mRNA transcript by transcriptional elongation. It has been demonstrated that several families of RNA polymerase II elongation factors and nuclear proteins aid in this process and that this elongation stage is critical in the regulation of gene expression (Conaway, Shilatifard, Dvir *et al.*, 2000, Reines, Conaway & Conaway, 1996). Transcriptional elongation is regulated by the alteration of the RNA polymerase II catalytic site, by the modification of chromatin structure, phosphorylation of RNA polymerase II and by interactions with other transcriptional components (Kobor & Greenblatt, 2002, Maldonado & Reinberg, 1995, Reines *et al.*, 1996, Shilatifard, 1998). Termination is believed to occur at a point after the end of the open reading frame of the protein being transcribed, however, it is still not clear if a defined termination signal actually exists, or if the dissociation of a transcription factor acts to destabilise the complex and cause the release of RNA polymerase II (Reeder & Lang, 1997, Tjian, 1995, von Hippel, 1998).

1.10.3 Transcription factors

Eukaryotic transcription factors are proteins, which bind specifically to DNA regulatory regions and by doing so they influence the degree of transcription of specific genes (Mitchell & Tjian, 1989). Transcription factors are made up of a number of different domains, each of which has a distinct function; these include DNA binding domains, dimerisation domains, activation domains, and repressor domains (Dynan & Tjian, 1985, Tjian, 1995). DNA binding domains act to couple the protein to its DNA binding motif and these include helix-turn-helix domains (homeodomain transcription factors), zinc finger domains (SP-1) and basic domains (bZIP factors) (Harrison, 1991).

Dimerisation domains aid in the coupling of dimeric transcription factors such as the bZIP family and the MyoD family of helix-loop-helix factors (McKnight, 1991, Muller & Schaffner, 1990, Rhodes & Klug, 1993). Transcription activation and repressor domains are the most important aspects of transcription factors, and it is through these domains that the function of the individual factors is realised (Herschbach & Johnson, 1993). The exact mechanisms by which transcription factors influence transcription are unclear, however, it is thought that they may alter chromatin structure to expose, or to bury specific DNA sequences (Cosma, Tanaka & Nasmyth, 1999, Kornberg & Lorch, 1999, Korzus, Torchia, Rose *et al.*, 1998, Ogryzko, Schiltz, Russanova *et al.*, 1996). Transcription factors may also interact with specific elements of the basal transcription complex to either promote or repress the level of transcription initiation (Laity *et al.*, 2001). In addition, it is well documented that different transcription factors are able to interact with each other, and they are able to do this even when they are bound to distant regions of DNA (Seto, Lewis & Shenk, 1993, Tjian, 1995). This may well represent another method of transcriptional regulation in eukaryotes and adds to the increasing complexity of gene expression control.

1.10.4 Transcription factor binding motifs in the PrP gene promoter

The PrP promoter contains binding motifs for a variety of transcription factors, however, these motifs vary between the different mammalian species and the

exact position and number of certain motifs is also species-specific. The mouse PrP promoter contains three adjacent binding motifs for SP-1 and single motifs for the AP-1 and AP-2 transcription factors (Baybutt & Manson, 1997). The human PrP gene contains binding motifs for SP-1 (two adjacent), AP-1, AP-2, GATA transcription factors, nuclear factor of activated T cells (NF-AT) and for nuclear factor of IL-6 (NF-IL6) (Funke-Kaiser *et al.*, 2001, Mahal *et al.*, 2001). The bovine promoter contains an AP-2 motif and three adjacent SP-1 motifs, whilst the ovine PrP promoter contains two AP-2 binding motifs (Figure 1.8) (Inoue *et al.*, 1997, O'Neill *et al.*, 2003). A polymorphic change of C to G within the downstream ovine AP-2 motif creates a single SP-1 binding motif in Cheviot sheep (O'Neill *et al.*, 2003). Figure 1.8 shows the first 442 bases upstream of the ovine PrP promoter, the known transcription factor motifs are marked and the start sites used are those described by Westaway *et al.* (1994b).

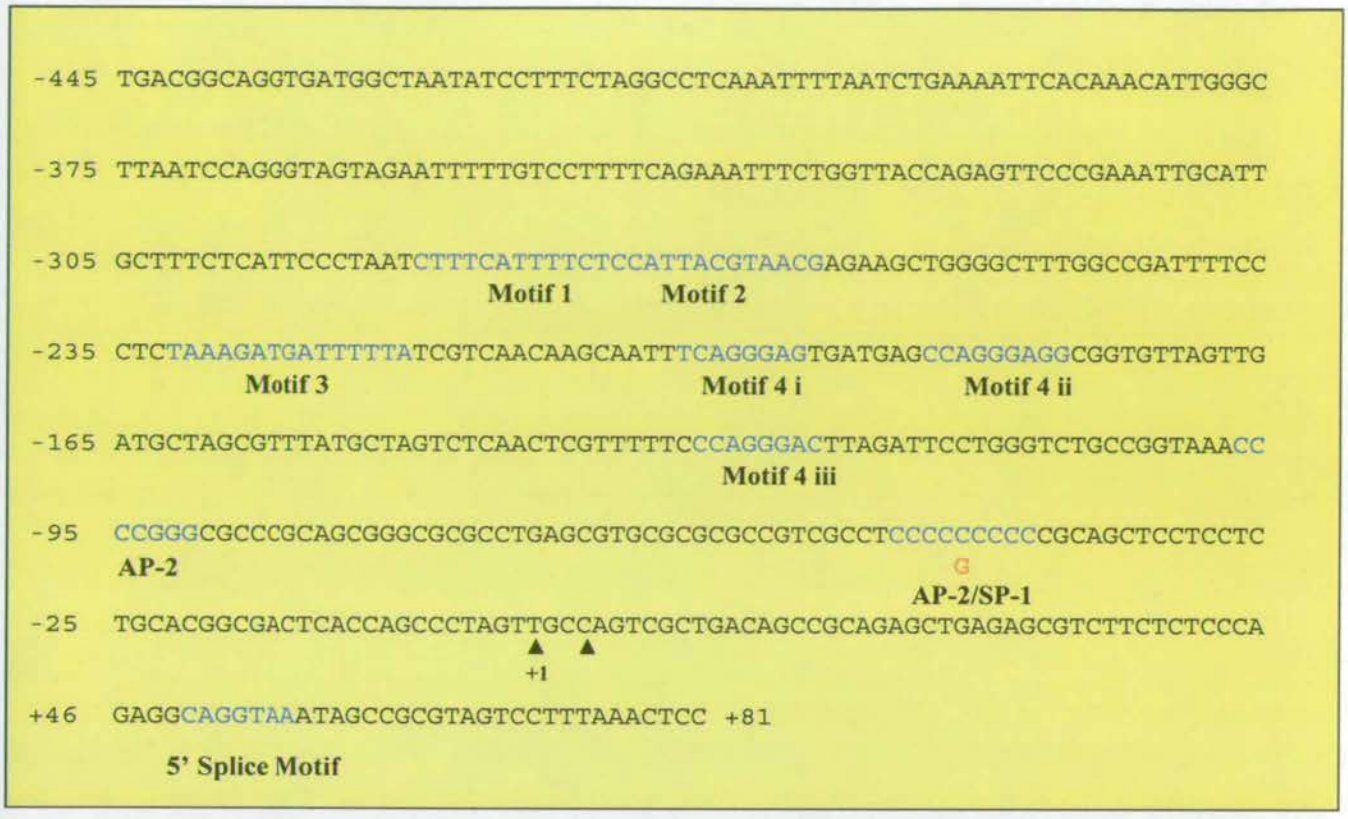


Figure 1.8 *Putative transcription factor binding motifs within the ovine PrP gene promoter*

Motifs 1-4 are conserved sequence elements found in ovine, murine, hamster, cattle and human PrP gene promoters, as identified by Westaway *et al.* (1994a) which may form binding sites for as yet unidentified transcription factors. Arrowheads indicate location of transcription start sites as experimentally shown by Westaway *et al.* (1994a). Suffolk sheep PrP gene promoter sequence from -445 to +81 bp, numbering based on the use of the 5' transcription start site (Genbank = U67922). Also highlighted are a potential binding site for the AP-2 transcription factor and a polymorphic AP-2/SP-1 site seen in Cheviot sheep (O'Neill *et al.*, 2003). Polymorphic or species specific base changes are indicated by base change (different colour) under the wild type (Suffolk, Genbank = U67922) sequence.

1.10.5 PrP gene conserved promoter motifs

Westaway *et al.* (1994a) discovered the presence of four motifs that are conserved within sheep, mouse, rat, human and Syrian hamster PrP genes which may be candidate binding motifs for as yet unspecified transcription factors. Motifs 1 and 3 (Figure 6.1) contain a high proportion of adenine (A) and thymine (T) residues, whilst motifs 2 and 3 form imperfect palindromic sequences, similar to binding

motifs of dimeric transcription factors (Figure 6.1). Motif 2 shares homology with the human bZIP repressor protein consensus sequence (Cowell, Skinner & Hurst, 1992, O'Neill *et al.*, 2003). Motif 4 is repeated in degenerate copies in both the sheep and Syrian hamster PrP promoters and has been identified as sharing homology with certain muscle-specific genes and with the binding motif of the lymphocyte-specific DNA-binding protein (LyF-1) (Hahm, Ernst, Lo *et al.*, 1994, Westaway *et al.*, 1994a). Experiments carried out at the NPU have revealed polymorphisms within the ovine PrP motif 1, which makes it especially interesting to investigate its role in PrP gene regulation and therefore scrapie disease control (O'Neill *et al.*, 2003). In order to understand the mechanisms regulating PrP gene expression it is vital to define the role of these transcription factor binding motifs, and to identify candidate binding factors for the conserved motifs in a variety of cell types.

1.11 Aims and objectives

Natural scrapie must start as an infection in peripheral tissues, and how that infection proceeds may be determined by the location and amount of PrP^c expressed on specific cell types. It is hypothesised that firstly the presence of small differences in PrP^c expression levels could lead to measurable differences in the survival time, or scrapie pathology of individual sheep. Secondly, that these effects will be especially important in the periphery as the location of infectivity uptake and transport to the CNS. Thirdly, that the temporal and spatial regulation of these PrP^c expression differences may be linked to the PrP gene promoter and/or 3' untranslated region (3'UTR) and fourthly, that polymorphisms in the regulatory gene regions may lead to the genetic variability of the phenotypes proposed in propositions 1 to 3. The overall aims of this thesis were to investigate the regulation of ovine PrP gene expression, and to provide further insights into the role of the PrP gene non-coding regions in this regulation. These aims were addressed by answering the following questions:

1. At what level is endogenous PrP^c expressed in a series of ovine cell lines derived from CNS and peripheral tissues?

The ovine cell lines were to be used in transient transfection experiments using ovine PrP mini-gene constructs. In order to determine if the detection of PrP^c protein expressed from the transfected constructs was to be reliable it was first important to establish the endogenous level of PrP^c expression in the ovine cell lines in comparison with the established mouse N2a cell line to be used as a control.

The following objectives were set:

- To determine if endogenous PrP^c expression within the ovine cells could be detected by immunoprecipitation and Western blot analysis.
- To verify that the detected protein has the characteristics of PrP^c, i.e. the expected molecular weight and glycosylation pattern.
- To compare ovine brain and peripheral cell lines
- To compare PrP^c expression from the ovine cell lines to that seen in the mouse N2a neuroblastoma cell line.

2. Can alternative polyadenylation of ovine PrP mRNA, as observed in sheep tissues, be associated with the control of PrP^c expression?

In sheep the PrP gene mRNA is alternatively polyadenylated producing two different sized mRNA transcripts, 2.1 & 4.6 kb. To date, the level of PrP^c protein expression attributable to each of these transcripts is not known. In order to determine the role of alternative polyadenylation in ovine PrP^c expression, a series of ovine PrP mini-gene constructs were produced which differed only in the availability of polyadenylation signals. The ovine cell lines were transfected with these constructs with the objective of analysing the level of recombinant PrP^c expression from each of the cell lines.

The following objectives were set:

- To label the recombinant PrP with the 3XFLAG epitope within the open reading frame of the PrP gene in order to be able to distinguish between endogenous and recombinant PrP expression.
- To determine if epitope-tagged PrP^c protein can be expressed in ovine cell lines from a transgene, which is able to encode various mRNA transcripts.
- To investigate whether alternatively polyadenylated PrP mRNA in transiently transfected cell lines can be associated with changes in the levels of epitope-tagged PrP^c protein.
- To verify that the 2.1 kb mRNA transcript is actively translated and to investigate whether its level of translation varies when compared to the 4.6 kb mRNA transcript.
- To analyse the possible connection between transcript translation level and specific RNA binding factors that may prevent or limit the expression of one or the other transcript in the brain

3. Do sequence specific transcription factors bind to sequence motifs within the ovine PrP promoter?

Sequences within the ovine PrP promoter, including the four conserved motif sequences identified by (Westaway *et al.*, 1994a), may form potential binding sites for sequence specific transcription factors and thus could act in the control of gene expression. The aim was to establish if these sequences are bound by transcription factors present in nuclear extracts prepared from ovine cell cultures and to consider if the binding of these factors indicated potential mechanisms by which the expression of the PrP gene could be controlled.

The following objectives were set:

- To identify potential binding sites for sequence specific transcription factors in the ovine PrP gene promoter sequence, using a variety of online and offline sequence analysis packages.

- To assess the binding of transcription factors present in nuclear extracts derived from ovine and murine cell lines using gel shift and DNase I Footprinting techniques.
- To identify the factors which bind to motifs within the ovine PrP promoter with antibodies specific for the candidate proteins using the gel super-shift assay and to elucidate potential pathways by which, PrP gene expression may be regulated.

4. Do single nucleotide polymorphisms within the ovine PrP promoter affect transcription factor binding?

A number of single nucleotide polymorphisms (SNPs) have been identified within the ovine PrP gene promoter. These polymorphisms may affect the binding specificity of transcription factors for the ovine PrP gene promoter, by either altering the composition of existing motifs or by creating novel motifs. It is hypothesized that differences in the binding of transcription factors to the ovine PrP promoter could result in different levels of PrP gene expression and so the aim was to determine if these polymorphisms affect the binding affinity of sequence-specific transcription factors by comparing mutant and wild type sequences in gel shift assays.

The following objectives were set:

- To establish whether or not SNPs affect the composition of DNA binding motifs within the ovine PrP promoter.
- To determine the effect of these SNPs on binding activity to oligonucleotides containing the wild type and mutant promoter motif sequences using gel shift assays.

5. Is the binding of transcription factors to the ovine PrP promoter cell/ tissue specific?

Ovine cell lines derived from Cheviot sheep of both known scrapie susceptible and resistant genotypes are available at the NPU. The promoter sequences are identical however there may be other features of the make up of these

cell lines which could affect PrP gene expression, for example different mixtures of transcription factors may be available in the scrapie susceptible and resistant cell lines. There may also be tissue-specificity in the mixture of available transcription factors in cell lines/ tissue samples of central nervous system (neuronal) and peripheral origin and these differences may affect the regulation of PrP gene expression.

The following objectives were set:

- To determine if there are tissue-specific differences in the availability of transcription factors between nuclear extracts prepared from the ovine cell lines derived from neuronal and peripheral tissues and also between nuclear extracts prepared from ovine and murine tissue samples of neuronal and peripheral origin.
- To determine if there are differences in the mixture of available transcription factors between the cell lines derived from sheep of known scrapie susceptible and resistant genotypes.

Chapter 2: Materials & Methods

2.1 General chemicals and solutions

All solutions used in this thesis were prepared with high quality chemicals and were sterilised by autoclaving when necessary. The water used to make up solutions was obtained from a Milli-QUF system (Millipore, UK), and was further autoclaved before use when necessary. All temperature-controlled incubations were performed in a hot block (Techne Dri-Block® DB-3), with thermometer monitoring, unless otherwise stated. All centrifugations were carried out at full speed (14,000 rpm) in and Eppendorf 5417C centrifuge unless otherwise stated. Standard solution recipes are detailed in **Appendix A.1**.

2.2 General methods for DNA cloning

The experimental procedures described below were performed according to the methods described in Sambrook & Russell (2001) or following the manufacturer's instructions, unless stated otherwise.

2.2.1 Restriction enzyme digestions

Restriction enzyme digestions were prepared as follows: 0.2-1 μg sample DNA, 10X restriction enzyme buffer (Roche, UK) and 10 units of restriction enzyme were mixed in a 0.5 ml microcentrifuge tube (Corning, UK) with sterile dH_2O to a total volume of 30 μl. A brief centrifugation at full speed was followed by incubation at the appropriate temperature (mostly 37 °C, but enzyme dependent) for 1-2 hours. The digestion was stopped by the addition of 5X gel loading buffer and results were visualised by agarose gel electrophoresis (Section 2.2.6).

2.2.2 DNA polymerase I large fragment (Klenow)

The Klenow enzyme is the large fragment of DNA polymerase I. Klenow catalyses the addition of mononucleotides from deoxynucleosides-5'-phosphates to

the 3'-hydroxyl terminus of a DNA template and is also used for the removal of 3'-overhangs. For the removal of a 3'-overhang, 1 unit of Klenow (2 units/μl, Roche, UK) was added for every μg of DNA in a 0.5 ml microcentrifuge tube, along with 1 μl of 10X reaction buffer (Roche, UK) and dH_2O to 10 μl. The reaction was mixed by vortexing and incubated at room temperature for 20 minutes. In order to fill a 5'-overhang, 40 mM of each dNTP (Roche, UK), and 1 unit of Klenow was added for every μg of DNA, mixed with 1 μl 10X reaction buffer (Roche, UK), dH_2O to a total of 10 μl and incubated for 1 hour at 37 °C. Klenow enzyme was inactivated by heating to 75 °C for 10 minutes.

2.2.3 Dephosphorylation of 5'- phosphates of DNA

Shrimp alkaline phosphatase (SAP) catalyses the dephosphorylation of 5'-phosphates from DNA and is used to dephosphorylate restricted cloning vectors in order to prevent re-ligation. For overhang cloning, SAP (Roche, UK) was added directly to the completed restriction enzyme digestion (Section 2.2.1). Three point six units of SAP (1 unit/μl) were added to the 30 μl restriction digestion along with 3.6 μl 10X dephosphorylation buffer and the mixture was incubated at 37 °C for 10 minutes. For blunt-end cloning, SAP was added to the completed restriction digestion as above and incubated for 60 minutes at 37 °C. SAP was inactivated by heating to 65 °C for 15 minutes.

2.2.4 Covalent ligation of DNA ends

T4 DNA ligase is an ATP-dependent enzyme, which covalently joins blunt or compatible cohesive DNA ends, as well as filling in nicks in double-stranded DNA. Twenty five ng of linearised (Section 2.2.1), dephosphorylated (Section 2.2.3) plasmid DNA, 100-200 ng of insert DNA, 1 unit of T4 DNA ligase (1 unit/μl, Roche, UK) and 1 μl 10X T4 DNA ligase buffer were mixed in a 0.5 ml microcentrifuge tube with sterile dH_2O to a total volume of 10 μl. The reaction was then incubated at 12 °C overnight in a thermal cycler (Techne PHC-3).

2.2.5 Labeling of DNA with ^{32}P

T4 polynucleotide kinase (T4 PNK) catalyses the transfer of the γ-phosphate from ATP to the 5'-terminus of polynucleotides or mononucleotides and is used in the radiolabeling of probes for differential hybridisation experiments. Two µl of oligonucleotide (2.5 pmol/µl), 1 µl of [γ-^{32}P]-ATP (3,000 Ci/mmol at 10 mCi/ml) (Amersham, UK), 10X kinase buffer (Promega, UK) and 5 units of T4 PNK (5-10 units/µl, Promega, UK) were mixed in a 0.5 ml microcentrifuge tube with sterile dH_2O to a total volume of 10 µl. The reaction was mixed and incubated at 37 °C for 10 minutes in a hot block in a lead-shielded vessel.

2.2.6 Agarose gel electrophoresis

For analysis of PCR products and restriction digestion samples the method described by Helling, Goodman & Boyer (1974) was used. Samples were prepared for loading by the addition of 1/5th the sample volume of 5X gel loading buffer, mixed and loaded directly onto a 1 % agarose gel. The gel was run at 120 V, 25 mA in 1X TBE buffer until sample separation was achieved. DNA bands were visualised over UV light using an UV trans-illuminator (Appligene, UK).

2.2.7 Isolation of DNA bands from agarose gels

Working over UV light, the required band (~0.1g) was cut from the agarose gel (Section 2.2.6) using a clean disposable sterile scalpel and placed into a sterile 1.5 ml microcentrifuge tube (Corning, UK). The DNA was then extracted from the agarose using a gel extraction kit (Qiagen, UK) following the manufacturer's protocol.

2.2.8 Transformation of JM109 competent *E. coli* cells

In a 1.5 ml microcentrifuge tube 30 µl of JM109 competent cells (Promega, UK) and 3 µl of a 10 µl ligation reaction (Section 2.2.2) were mixed and incubated on ice for 30 minutes. The cells were heat-shocked at 42 °C in a hot block for 1.5 minutes and then incubated on ice for a further 10 minutes. LB broth (500 µl, Roche,

UK) was added and shaken for 1 hour at 37 °C in a shaking incubator (Brunswick Series 25). Four 120 µl aliquots of transformed competent cells were plated out onto ampicillin supplemented agar (1 µl/ml) and incubated overnight at 37 °C (Gallenkamp Size 1 economy incubator). For blue/white selection transformed competent cells were spread onto ampicillin positive IPTG/X-Gal agar plates and incubated as above.

2.2.9 Selection of bacterial colonies for amplification

Single colonies were selected and placed into 4 ml LB amp broth (1 µl/ml ampicillin) in a 15 ml centrifuge tube and incubated overnight with shaking at 37 °C. For blue/white selection, only white colonies; those with an interrupted Lac Z gene, indicating that the desired fragment had been incorporated, were selected.

2.2.10 Plasmid DNA minipreps by alkaline lysis method

For the small scale preparation of DNA from transformed *E. coli* cells the following protocols were used (Birnboim & Doly, 1979, Ish-Horowicz & Burke, 1981). In a 1.5 ml microcentrifuge tube 1 ml of a bacterial culture of transformed *E. coli* cells (Section 2.2.8) was spun for 1 minute at full speed. The supernatant was discarded and a further 1 ml of bacterial culture was loaded into the same tube. The tube was spun again as detailed above and the supernatant was again discarded. The cells were lysed by the addition of 100 µl miniprep solution A, producing a bacterial suspension, which was incubated at room temperature for 10 minutes. Proteinaceous material was precipitated by the addition of 200 µl miniprep solution B. The solution was mixed by vortexing and incubated on ice for 5 minutes. Finally, nucleic acids were precipitated by the following method: miniprep solution C (150 µl) was added and the sample was mixed, incubated on ice for 10 minutes and spun at full speed for 1 minute. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube and 45 µl of sodium acetate (NaAc, pH 5.2) and 450 µl of iso-propanol were added. The tube was vortexed briefly and placed at –20 °C for 1 hour. The preparation was then spun at full speed for 10 minutes, the supernatant was discarded and the pellet

containing nucleic acids was washed with 500 µl ice-cold 70 % ethanol, vortexed briefly and spun at full speed for 5 minutes. Excess ethanol was discarded and the tube was blotted dry and air dried for about 1 hour at room temperature. The pellet was dissolved in 50 µl RNase solution to remove residual RNA, incubated at room temperature for 20 minutes and stored at –20 °C.

2.2.11 Ethanol precipitation of plasmid DNA

To purify the DNA preparation (Section 2.2.10) the sample was ethanol precipitated as follows: Two times the sample volume of 100 % ethanol was added to the plasmid DNA preparation, the solution was vortexed briefly and incubated at –20 °C for at least 30 minutes. The sample was spun at full speed for 10 minutes and the supernatant was discarded. The DNA pellet was dissolved in 50 µl of RNase solution to remove residual RNA, incubated at room temperature for 20 minutes and stored at –20 °C.

2.2.12 Plasmid DNA maxipreps by bulk alkaline lysis method

For the large scale preparation of DNA from transformed *E. coli* cells the following protocols were used (Birnboim & Doly, 1979, Ish-Horowicz & Burke, 1981). A starter culture of 4 ml ampicillin LB amp broth was inoculated with a single colony of transformed *E. coli* cells and incubated overnight at 37 °C with shaking (Section 2.2.8). This culture was added to 300 ml LB amp broth and incubated as before. The culture was spilt into 2 x 250 ml GSA bottles (Beckman, USA), centrifuged at 6,000 rpm for 10 minutes at 4 °C (Beckman JL-21) and the supernatant was discarded. The pellets were dissolved in 30 ml miniprep solution A (cell lysis solution) plus 0.1 g of lysozyme (10 µg/ml, Sigma, UK) and the bottles were incubated at room temperature for 10 minutes. Sixty ml of miniprep solution B was added to precipitate proteinaceous material and the bottles were mixed well and incubated on ice for 5 minutes. Miniprep solution C (30 ml) was added to precipitate nucleic acids, and after mixing, the bottles were again incubated on ice for 15 minutes and spun at 6,000 rpm for 10 minutes at 4 °C. The supernatants were pooled,

filtered through gauze into a 500 ml cylinder and 0.6 times the sample volume of iso-propanol was added. The contents were poured into a clean GSA bottle and spun at 8,000 rpm for 10 minutes at 4 °C (Beckman JL-21). The supernatant was discarded, residual liquid was blotted from the bottle neck and the pellet was air dried for 1 hour. The pellet was dissolved in 4 ml of TE buffer (pH 7.4) and transferred to a glass tube (Corex, USA), to which 4 ml of phenol/chloroform/iso-amyl alcohol (25:24:1, Sigma, UK) was added. The tubes were covered in Parafilm (American National Can, USA), mixed carefully by slow vortexing and spun for 5 minutes at 8,000 rpm at 4 °C. The upper aqueous layer was removed to a fresh glass tube. To this tube 1/10 the sample volume of 3M NaAc (pH 5.2) and 2.5 times the sample volume of absolute alcohol were added, the solution was mixed and placed at –20 °C for 1 hour. The tube was spun at 8,000 rpm for 5 minutes at 4 °C, the supernatant was discarded and the pellet was air dried and dissolved in 2 ml TE buffer. A caesium chloride (CsCl) gradient was prepared in 5 ml Quickseal tubes (Beckman, UK) by adding 4.75 g CsCl to 2 ml DNA preparation, along with 100 µl ethidium bromide solution (0.5 µg/ml). The tubes were then filled to the top with TE buffer, sealed using a Beckman tube sealer and spun overnight at 80,000 rpm at 20 °C (Beckman Optima TLX Ultracentrifuge). The lower plasmid band (~1 ml) of the CsCl preparation was removed with a wide bore needle syringe and made up to 5 ml with TE buffer in a 15 ml plastic centrifuge tube. The ethidium bromide was removed by adding 5 ml butan-1-ol, mixing by inversion and the removal of the top DNA layer to a fresh tube. This wash procedure was repeated three times or until all ethidium bromide was removed. Ethanol precipitation (Section 2.2.11) was used to recover the DNA followed by centrifugation at 8,000 rpm for 10 minutes at 4 °C, the pellet was air dried, dissolved in 1 ml of TE buffer (pH 7.4) and stored at –20 °C.

2.2.13 Spectrophotomeric determination of DNA sample concentration and purity

DNA sample concentration was determined by spectrophotomeric analysis. An aliquot of the DNA sample (8 µl) was added to 800 µl of dH_2O, mixed well and transferred to a silica spectrophotometer cuvette (Sigma, UK). Absorbance at 260 nm

and 280 nm was determined using a UV spectrophotometer (Beckman DU®650). DNA concentration (μg/ml) was calculated by multiplying the absorbance value at 260 nm by 5000 to take into account the dilution value. DNA purity was determined by dividing the absorbance value at 260 nm by the value at 280 nm. A value greater than 1.9 was considered as being a good quality sample, with minimal protein contamination.

2.2.14 Polymerase chain reaction (PCR)

First described by Kary Mullis in 1986 the polymerase chain reaction (PCR) is a process by which *Thermus aquaticus* DNA polymerase I (Taq DNA polymerase I) synthesises a complementary strand to a given DNA strand (Mullis, Faloona, Scharf *et al.*, 1986). This reaction takes place in a mixture containing dNTPs and 2 complimentary primers, which flank the target DNA sequence. The mixture is first denatured to produce single stranded template DNA containing the target sequence then cooled to allow the primers anneal to their complementary sequences. The primers are then extended by the Taq polymerase into newly synthesised complementary strands. As each new double strand separates to two individual templates, multiple cycles allow the target DNA to multiply exponentially. In just 20 cycles of PCR, target template can be amplified by a million-fold. The concentration of each primer (Table 2.1, MWG, DE) used for PCR reactions was 25 pmol per 50 μl reaction and dNTPs (Roche, UK) were used at a final concentration of 200 mM. The following reaction was used: 1 unit Taq polymerase (1 unit/μl, Roche, UK), 0.4 μl 25 mM dNTPs (Roche, UK), 10X Taq buffer (Roche, UK), 1 μl each primer (25 pmol, MWG, DE), 30 ng plasmid DNA (10 ng/μl) were mixed in a 0.5 ml microcentrifuge tube with sterile dH_2O to a final volume of 50 μl. Negative reactions with no DNA template added were used as controls. PCR reactions were incubated in a PCR thermal Cycler (Techne PHC-3). The following is an example cycle used for PCR reaction: DNA was denatured at 94 °C for 1 minute, primers and template DNA were annealed at 60 °C (temperature optimised for particular primer) for 1 minute and new DNA strand was synthesised behind the primers on each template strand at 72 °C for

2 minutes. Commonly this cycle was repeated at least 30 times. PCR reactions were analysed by agarose gel electrophoresis (Section 2.2.6) and stored at –20 °C.

2.2.15 Pre-ligation treatment of PCR products

PCR products (Section 2.2.14) were cleaned and prepared for ligation into the chosen plasmid by the following method. Forty µl PCR product, 2 µl 10 % SDS (Sigma, UK), 1 µl 0.25 M EDTA (pH 8,) and 1 µl (20 mg/ml) proteinase K (Sigma, UK) were mixed in a 0.5 ml microcentrifuge tube and incubated at 37 °C for 30 minutes. To precipitate DNA, 4.5 µl of NaAc (pH 7) and 125 µl absolute alcohol was added and incubated overnight at –20 °C. The precipitated DNA sample was spun at full speed for 10 minutes and the supernatant was discarded. The pellet was washed in 500 µl ice cold 70 % ethanol and vortexed briefly. The sample was spun at full speed for 5 minutes, the supernatant was discarded, the pellet was blotted dry and air dried for 1 hour at room temperature. The dried pellet was dissolved in 20 µl dH_2O and stored at -20 °C. Pre-ligation treated PCR product was then ligated into the appropriate vector as described in Section 2.2.4.

2.2.16 Reverse Transcriptase – PCR (RT-PCR) with Superscript II

Superscript II (Invitrogen, UK) was used to synthesise complementary DNA (cDNA) from total RNA extracts isolated from ovine cell lines (Section 2.5.8) following the manufacturer's protocol. Briefly, 1-5 µg total RNA (Section 2.5.8), 1 µl sequence specific primer (2 pmol) (Table 2.1), 1 µl 10 mM dNTP mix (Roche, UK) were mixed in a 0.5 ml microcentrifuge tube with dH_2O to 12 µl. The reaction was heated to 65 °C for 5 minutes, the contents were collected by brief centrifugation at full speed and chilled on ice. To the reaction was added 4 µl 5X first strand buffer and 2 µl 0.1 M DTT (Invitrogen, UK). The contents were mixed gently and incubated at 42 °C. Two hundred units of Superscript II (200 units/µl, Invitrogen, UK) were added, the contents were mixed by pipetting and incubated at 42 °C for a further 50 minutes. The reaction was inactivated by heating to 70 °C for 15 minutes.

The cDNA prepared was used as a template for PCR amplification as described in Section 2.2.14.

Primer name	Target sequence (Suffolk sheep Genbank = U67922)	DNA strand	Primer sequence
AG23	3' end of ovine PrP gene promoter (5675-5696 bp)	Top	5'CTGACAGCCGCAGA GCTGAGAG'3
580SH	5' end of ovine PrP gene ORF (23125-23144 bp)	Bottom	5'GTGGTGGTGACTGT GTGTTG'3
643	5' end of ovine PrP gene ORF (22902-22921 bp)	Bottom	5'GCTCCACCACTCGCT CCATT'3
13741	5' end of ovine PrP gene ORF (22423-22447 bp)	Bottom	5'GAGGCCTGAGGTGG ATAGCGGTTGC'3
Flag Forward	5' end of 3XFLAG™ DNA sequence	Top	5'TCTCTACCCGGGAG ACTACAAAGAC'3
Flag Reverse	3' end of 3XFLAG™ DNA sequence	Bottom	5'CTAAATCCCGGGTA CTTGTCATCGTC'3

Table 2.1 *PCR & RT-PCR primer sequences*

Sequence used for primer design was the Suffolk sheep sequence (Genbank = U67922). Primers were designed to recognise sequences (top = top strand, bottom = bottom strand) within the sheep PrP gene open reading frame (ORF), the sheep PrP gene promoter or the 3XFLAG DNA sequence (Sigma, UK).

2.3 Sanger (dideoxy mediated) chain termination sequencing of DNA

2.3.1 Chain termination sequencing reaction

This technique which was first described by Sanger, Nicklen & Coulson (1977) involves the *in vitro* synthesis of a DNA strand by a DNA polymerase with the use of a specifically primed single-stranded DNA template (Section 2.3.2). The

first step is a labeling procedure in which the primer is extended using limiting concentrations of deoxynuleotides (dNTPs), including a radioactively labelled dATP. These primer extensions form a population of chains of various lengths from several to hundreds of nucleotides. The second step involves the addition of chain terminating nucleotide analogue, 2',3'-dideoxynucleoside-5'-triphosphates (ddNTPs). These analogues lack the 3'-OH group required for DNA chain elongation and processive DNA synthesis occurs until all extending chains terminated by a ddNTP. When mixtures of dNTPs and one of the four ddNTPs are used polymerisation will be terminated in a fraction of the population of chains at each site where the ddNTP can be incorporated. Using four separate reactions, each with a different ddNTP, it is possible to obtain complete DNA sequence information (Section 2.3.3). Sequencing reactions are halted by the addition of EDTA and formamide, denatured by heating, separated by high resolution denaturing gel electrophoresis (Section 2.3.4) and visualised by autoradiography.

2.3.2 Preparation of single strand DNA (ssDNA) template

Twenty µl of miniprep DNA (2 mg/ml) (Section 2.2.10), 2 µl of 2M NaOH and 2 µl of 2 mM EDTA were mixed in a 0.5 ml microcentrifuge tube and incubated for 30 minutes at 37 °C. The reaction was neutralised by the addition of 2.5 µl of 3M NaAc (pH 5.2). DNA was precipitated by adding 100 µl of absolute alcohol and incubated at –70 °C for 15 minutes. The DNA was pelleted for 10 minutes at full speed, the pellet was washed with 500 µl ice cold 70 % ethanol and spun at full speed for 5 minutes before discarding the supernatant. The pellet was blotted and air dried for 1 hour, before being dissolved in 25 µl TE buffer (pH 7.4) and stored at –20 °C.

2.3.3 Chain termination sequencing protocol

To prepare the annealing mixture the following were added together in a 0.5 ml microcentrifuge tube: 7 µl single stranded plasmid DNA (Section 2.3.2), 2 µl reaction buffer (Amersham, UK) and 1 µl sequencing primer (1 pmol/µl) (Table 2.2).

The mixture was heated for 2 minutes at 65 °C in a water bath and allowed to cool slowly to 35 °C before being centrifuged briefly at full speed and further chilled on ice. Whilst the annealing mixture was cooling the termination mixtures (ddNTPs, Amersham, UK) were prepared by the addition of 2.5 µl of each termination mix (G, A, T and C) into labelled microcentrifuge tubes and incubated at 37 °C. The labeling mix (Amersham, UK) was prepared by diluting the stock 1 in 5 with dH_2O. The Sequenase (Version 2.0, Amersham, UK) was prepared by diluting 1 in 10 with Sequenase buffer (Amersham, UK). The labeling reaction was prepared by adding the following to the chilled DNA mix (10 µl): 1 µl 0.1 M DTT (Amersham, UK), 2 µl diluted labeling mix, 0.75 µl [^{s}35] dATP (Amersham, UK), 1 µl manganese buffer (Amersham, UK) and 2 µl diluted sequenase version 2. The labeling reaction was incubated at room temperature for 2 minutes, 3.5 µl was transferred to each of the four termination mixtures (G, A, T and C), mixed by vortexing and incubated at 37 °C for 5 minutes. The reactions were stopped by the addition of 4 µl of stop solution, which contains EDTA and formamide (Amersham, UK). Samples were heated to 75 °C for 2 minutes immediately prior to loading onto sequencing gel (Section 2.3.4).

Primer name	Target (Suffolk sheep Genbank = U67922)	DNA strand	Primer sequence
Flag Forward	5' end of 3XFLAG™ DNA sequence	Top	5'TCTCTACCCGGGAG ACTACAAAGAC'3
Flag Reverse	3' end of the 3XFLAG™ DNA sequence	Bottom	5'CTAAATCCCGGGTA CTTGTCATCGTC'3
AG28	3' end of ovine PrP gene promoter (5680-5702 bp)	Top	5'AGCCGCAGAGCTGA GAGCGTCTT'3
B007	5' end of ovine PrP gene ORF (22335-22356 bp)	Bottom	5'GCTTCTTGCAGAGG CCCACGTC'3
Promseq1	3' end of ovine PrP gene promoter (5636-5655 bp)	Top	5'TCCTCTGCACGGCG ACTCAC'3
Promseq2	3' end of ovine PrP gene promoter (5646-5655 bp)	Top	5'GGCGACTCACCAGC CCTAGT'3
Promseq3	3' end of ovine PrP gene promoter (5546-5565 bp)	Top	5'AGATTCCTGGGTCT GCCGGT'3
FP1	5' end of ovine PrP gene promoter (5226-5245 bp)	Top	5'TGACGGCAGGTGAT GGCTAA'3

Table 2.2 *Sequencing primers used to sequence Cheviot PrP promoter sequence and PrP mini-gene construct DNA*

Sequence used for primer design was the Suffolk sheep sequence (Genbank = U67922). Primers were designed to recognise sequences (top = top strand, bottom = bottom strand) within the sheep PrP gene open reading frame (ORF), the sheep PrP gene promoter or the 3XFLAG DNA sequence (Sigma, UK).

2.3.4 Denaturing gel electrophoresis for sequencing

A 3 µl aliquot of each sample was loaded onto a 4 % acrylamide gel, the gel was run for 20 minutes at 2,000 V, 50 watts, then run at 2,000 V, 35 watts for a further 1 hour. In order to remove urea the gel was placed into a bath with 5 % glacial acetic acid for 20 minutes. The gel was then transferred to a sheet of Whatman 3MM filter paper, wrapped in cling film and dried on a gel dryer at 80 °C for 1.5 hours. Once dried the gel was transferred to a cassette (Genetic Research Instrumentation Ltd., UK) and exposed to Kodak Biomax MR-1 X-ray film (Kodak, UK) for 24 hours at room temperature.

2.3.5 Automated sequencing

Automated sequencing was performed using an ABI Prism 377 automated sequencer. Prior to sequencing, PCR or RT-PCR products (Sections 2.2.14 and 2.2.16) of the DNA sequence to be sequenced were purified and concentrated using a Microcon YM-100 filter (Millipore, USA). To the filter, 360 µl of TE buffer and 40 µl PCR or RT-PCR product was added, the filter was then spun at 510 x g (Eppendorf 5417C) for 9 minutes, leaving approximately 20-30 µl of product on the filter. The filter was then inverted and placed in a clean 1.5 ml microcentrifuge tube, the tube was spun at full speed for 30 seconds to collect the sample, which was stored at –20 °C.

2.3.6 Automated sequencing reaction

The sequencing reaction consisted of the following components: 3 µl Big Dye kit (Microzone Limited, UK), 3 µl Better Buffer (Microzone Limited, UK), 2 µl sequencing primer (1 pmol/µl), 7 µl concentrated PCR product sample, for a total volume of 15 µl. The reaction was performed on a PCR thermal cycler (Hybaid, UK) with 25 cycles of the following programme: 96° C for 10 seconds, 50° C for 5 seconds and 60 °C for 4 minutes. To each reaction, 80 µl of isopropanol was added, the samples were then precipitated at room temperature for 20 minutes, prior to centrifugation at 3,000 rpm for 20 minutes at 4 °C. Samples were air dried, dissolved in 4 µl of Red Loading Buffer (Microzone Limited, UK) and denatured at 94° C for two minutes prior to loading onto a sequencing gel. The sequencing reaction utilises four separate fluorescent dyes, one for each base. The ABI sequencer then reads the signal from each dye using a laser, and in this way a sequence is derived from the sample as it is separated on the sequencing gel.

2.4 General methods for promoter analysis

2.4.1 Preparation of double stranded oligonucleotide probe

Complementary single stranded synthetic oligonucleotides (50 pmol/µl, MWG, DE) were annealed by mixing and incubating equal volumes (50 µl) of each strand in a hot block at 90 °C for 10 minutes. The hot block was then placed on the bench and the oligonucleotides were allowed to cool slowly to room temperature. The annealed oligonucleotides were diluted in dH_2O to a working concentration of 2.5 pmol/µl, aliquoted and stored at –20 °C. The sequences of the oligonucleotides used throughout this study are detailed in Table 2.3.

Putative transcription factor motif	Abbreviation	DNA binding motif oligonucleotide sequence (Top strand)
NC-1 (Promega, UK)	NC-1	5'ATTCGATCGGGGCGGGGCGAG'3
NC-2 (Promega, UK)	NC-2	5'GATCGAACTGACCGCCCGCGGCCCGT'3
Wild type STAT site	STAT-C	5'AGAGTTCCCGAAATTGCTTT'3
Variant STAT site	STAT-A	5'AGAGTTCCAGAAATTGCTTT'3
AP-1	AP-1	5'GGAGTGATGAGCCAGGGAGG'3
GATA-1	GATA-1	5'TGACGGCAGGTGATGGCTAA'3
EGR-1	EGR-1	5'GGGCGCCCGCAGCGGGCGCG'3
HSE-1	HSE-1	5'ATCTGAAAATTCACAAACAT'3
HSE-2	HSE-2	5'TGTCCCTTTTAGAAATTTCT'3
Upstream AP-2	uAP-2	5'TAAACCCCGGGCGCCCGCAG'3
Downstream AP-2 (Suffolk)	dAP-2s	5'CCTCCCCCCCCCCGCAGCTC'3
Downstream AP-2 (Cheviot)	dAP-2c	5'CCTCCCCGCCCCCGCAGCTC'3
Wild type motif 1	M1T	5'TAATCTTTCATTTTCTCCAT'3
Variant motif 1	M1C	5'TAATCTTCCATTTTCTCCAT'3
Ruminant motif 2	M2C	5'TTCTCCATTACGTAACGAGA'3
Non-ruminant motif 2	M2T	5'TTCTCCATTATGTAACGAGA'3
Motif 3	M3	5'CTCTAAAGATGATTTTTATC'3
Motif 4 (i)	M4i	5'GCAATTTCAGGGAGTGATGA'3
Motif 4 (ii)	M4ii	5'GATGAGCCAGGGAGGCGGTG'3
Motif 4 (iii)	M4iii	5'TTTTTCCCAGGGACTTAGAT'3
Variant M1 + Ruminant M2	M1C/M2C	5'TAATCTTCCATTTTCTCCATTACGTAACGAGA'3
Wild type M1 + Non-ruminant M2	M1T/M2T	5'TAATCTTTCATTTTCTCCATTATGTAACGAGA'3
Variant M1 + Non-ruminant M2	M1C/M2T	5'TAATCTTCCATTTTCTCCATTATGTAACGAGA'3
Wild type M1+ Ruminant M2	M1T/M2C	5'TAATCTTTCATTTTCTCCATTACGTAACGAGA'3
Humanised Ovine M1 + M2	hovM1/M2	5'GAGCCTTCCATTTTCTCGATTTCTCCATTACGTAACGGGGG'3
Human M1 + M2	hM1/M2	5'GAGCCTTTCATTTTCTCGATTTCTCCATTATGTAACGGGG'3

Table 2.3 *Binding motif oligonucleotide sequences used in gel shift & super-shift assays*

Oligonucleotides (20-40 bp) were designed to contain the appropriate putative transcription factor binding motif sequence along with the native flanking sequences (5' & 3'). Top strand sequence only is detailed in the table. Red letters indicate presence of polymorphic or species specific base changes

2.4.2 Labeling of double stranded DNA probe with [γ- ^{32}P] ATP

The double stranded oligonucleotide was labeled with [γ-^{32}P] ATP as detailed in Section 2.2.5. The reaction was stopped by the addition of 1 µl of 0.5M EDTA and diluted in 89 µl of TE buffer. The radiolabeled oligonucleotide was separated from the unincorporated nucleotide by chromatography through a G-50 ProbeQuant column (Amersham, UK) following the manufacturer's protocol. The radiolabeled oligonucleotide was counted in a 1.5 ml microcentrifuge tube using a Beta Scout 2007 liquid scintillation tester (Perkin-Elmer, USA). Only radiolabeled oligonucleotides with a count of 500,000 cpm or greater were used for gel shift assay analysis.

2.4.3 Preparation of nuclear extracts from cultured cells

Nuclear extractions were performed with the nuclear and cytoplasmic extraction reagents (NE-PER, Pierce, UK) following the manufacturer's protocol based on the method described by (Dignam, Lebovitz & Roeder, 1983). Cells were cultured to confluency in 75 cm^2 flasks yielding approximately 2 x 10^6 cells at 80 % confluency. Cultured cells were trypsinised (Section 2.5.3) and cells were pelleted by centrifugation at 3,000 x g for 2 minutes. The supernatant was removed and the pellet was re-suspended in 200 µl of ice cold Cytoplasmic extraction reagent I (CER I, Pierce, UK) buffer. The sample was vortexed for 15 seconds to fully re-suspend the pellet and incubated on ice for 10 minutes. At this stage 50 µl Complete Mini - proteinase inhibitor solution (Roche, UK) was added to the sample. Eleven µl of ice cold Cytoplasmic extraction reagent II (CER II, Pierce, UK) buffer was added, the sample was vortexed for 5 seconds, incubated for 10 minutes on ice and vortexed again for a further 5 seconds. The sample was centrifuged at 4 °C for 5 minutes at 16,000 x g in an Eppendorf 5415R centrifuge. The supernatant was discarded and the pellet containing nuclei was re-suspended in 50 µl of ice cold nuclear extraction reagent (NER, Pierce, UK) buffer, vortexed for 15 seconds and incubated on ice. The sample was vortexed for 15 seconds every 10 minutes for a further 40 minutes. The sample was then centrifuged at 4 °C for 10 minutes at 16,000 x g and the supernatant

(nuclear extract) was transferred to a clean pre-chilled microcentrifuge tube, aliquoted and stored at –70 °C until use.

2.4.4 Preparation of nuclear extracts from tissue samples

The extraction of nuclear extracts from tissue was carried out using the same protocol and reagents for cultured cells (Section 2.4.3) but with the following changes: Fresh or frozen tissue (50 mg) was homogenised using a pellet pestle motor (Kontes, UK). Five hundred µl CER I buffer was added to the tissue homogenate and this was mixed further with the pellet pestle motor until a homogenous solution was achieved. At this stage 50 µl Complete Mini - proteinase inhibitor solution was added. The same protocol for cultured cells was followed from this point (Section 2.4.3), however 27.5 µl of CER II buffer was added and the nuclei were re-suspended in 100 µl of NER buffer before being aliquoted and stored at –70 °C.

2.4.5 Determination of total protein concentration in nuclear extracts

Concentration of total protein in nuclear extracts was determined using spectrophotomeric analysis. In a 1.5 ml microcentrifuge tube, an 8 µl aliquot of the nuclear extract was added to 800 µl of dH_2O, mixed well and transferred to a silica spectrophotometer cuvette (Sigma, UK). Absorbance at 260 nm and 280 nm was determined using a UV spectrophotometer. The following calculation was performed to determine the total protein concentration (µg/µl):

Protein concentration (µg/µl) =

(Absorbance 280 nm x 1.55) – (Absorbance 260 nm x 0.76)

The value obtained above was multiplied by 100 to correct for the dilution factor, giving the total protein concentration in the nuclear extract in µg/µl. Concentrations of the nuclear extracts varied between the cell lines and tissue samples but were consistently between 5-20 µg/µl.

2.4.6 Gel shift (DNA binding) assay

The gel shift assay technique was first described by Freid & Crothers (1981) and Garner & Revzin (1981). The assay is used for the analysis of protein-DNA interactions. The assay separates free DNA from protein-DNA complexes by non-denaturing gel electrophoresis. It relies on the production of a positive signal, which is produced when a transcription factor binds specifically to its ^{32}P labeled DNA binding site, causing a band shift to occur. For each binding assay four separate reactions were prepared as follows:

Reaction 1 = Negative control: labelled probe only.
Reaction 2 = Positive control: labelled probe and nuclear extract (NE).
Reaction 3 = Specific competitor: labelled probe, NE & same unlabelled probe.
Reaction 4 = Non-specific competitor: labelled probe, NE & a different unlabelled probe.

The amount of NE added varied depending on the batch concentration and this was corrected for by varying the amount of nuclease free water added to each reaction. In general the amount of NE added was between 1-2 µl, providing between 10-20 µg of total nuclear protein per binding assay (Sections 2.4.3-2.4.4). Great care was taken to ensure that the same batch of nuclear extract was used for each reaction in a single binding assay. Gel shift assays were performed using the Gel Shift Assay Kit (Promega). Reactions consisted of 2 µl 5X binding buffer (250 mM NaCl, 50mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 2.5 mM DTT, 5 mM MgCl2, 20 % glycerol, 0.5 µg poly(dI·dC)·poly(dI·dC)), 1-2 µl (10-20 µg) NE and dH_2O to a total volume of 9 µl incubated at 25 °C for 10 minutes. Each reaction was prepared as detailed below:

Reaction 1 (Negative control)
7 µl Nuclease free water
2 µl Gel shift binding buffer 5X (Promega, UK)
0 µl Nuclear extract (NE)
9 µl Total volume

Reaction 2 (Positive control)
6 µl Nuclease free water
2 µl Gel shift binding buffer 5X (Promega, UK)
1 µl NE
9 µl Total volume

Reaction 3 (Specific competitor)
5 µl Nuclease free water
2 µl Gel shift binding buffer 5X (Promega, UK)
1 µl NE
1 µl unlabelled competitor oligo (2.5 pmol/µl)
9 µl Total volume

Reaction 4 (Non-specific competitor)
5 µl Nuclease free water
2 µl Gel shift binding buffer 5X (Promega, UK)
1 µl NE
1 µl unlabelled non-competitor oligo (2.5 pmol/µl)
9 µl Total volume

The above reactions were incubated at room temperature for 10 minutes before the addition of 1 µl ^{32}P labeled double stranded DNA probe (0.025 pmol, ~10,000-50,000 cpm/10-100 fmol) to give a total reaction volume of 10 µl (Section 2.4.2). The reaction was further incubated at 25 °C for 20 minutes prior to being loaded onto a 4 % non-denaturing acrylamide gel (Section 2.4.8). For competition experiments 100-fold molar excess of unlabeled (cold) competitor and/or non-competitor double stranded DNA probes were added.

2.4.7 Super-Shift Assay

Gel super-shift assays are a modification of the gel shift assay that involves the addition of an antibody that is specific for the DNA binding factor being analysed. If the factor in question is present in the gel shift reaction, then the initial shift will be further shifted or blocked by the binding of the antibody to the protein. Gel super-shift assays were performed as for gel shift assays, the specific antibody (1-2 µl of a 1 µg/µl antibody solution in PBS) was added immediately after the addition of the appropriate ^{32}P-labelled oligonucleotide. The super-shift reaction was incubated at room temperature for either 20 minutes or 45 minutes, or overnight at +4 °C prior to gel electrophoresis (Section 2.4.8).

2.4.8 Electrophoresis of DNA-Protein complexes

The assays were analysed by gel electrophoresis on a 4 % non-denaturing acrylamide gel. The gel was pre-run in 0.5X TBE buffer for 10 minutes at 250 V prior to loading, and after the samples were loaded on to the gel it was run at 250 V for one hour. The gel was transferred to a sheet of Whatman 3MM filter paper, covered in plastic wrap and dried on a gel dryer at 80 °C for 1 hour. The gel was then exposed to Kodak Biomax MS-1 X-ray film (Kodak, UK) for 1 hour, 12 hours and 72 hours respectively at –70 °C with an intensifying screen.

2.4.9 DNase I Footprinting

DNase I Footprinting works on the basis that the interaction of DNA with a regulatory protein protects that region of the DNA from the action of DNase I (Galas & Schmitz, 1978). The amount of DNase I added to the reaction is regulated so that it does not completely degrade the DNA probe, instead the aim is to cut each molecule at a single phosphodiester bond. If no protein is bound to the DNA then the result is a family of labelled fragments, differing in size by one base each, which appear as a ladder after gel electrophoresis. However, the bound protein protects certain bonds from degradation by DNase I, resulting in an incomplete family of labelled fragments. This shows up after gel electrophoresis as a gap in the ladder, allowing control sequences to be identified.

2.4.9.1 Preparation of DNA probe

The DNA probe used for DNase I Footprinting was the 0.5 kb Cheviot PrP gene promoter fragment as determined by O'Neill *et al.* (2003). This fragment contains the PrP gene 5'UTR from position 5226 to 5665, exon I from 5666 to 5717, and part of intron I from 5718 to 5748, using the numbering of the Suffolk PrP gene (GenBank accession number = U67922). This fragment was cleaved from the plasmid pNPU-110 with *Hind*III and *Eco*RI restriction enzymes (Section 2.2.1) to release a 526 bp fragment with 5' overhanging ends, which was agarose gel purified (Section 2.2.6).

2.4.9.2 Dephosphorylation of 5' ends

The 5' overhanging ends of the DNA probe were dephosphorylated using calf intestinal alkaline phosphatase (CIAP) (Promega, UK) in the following reaction: 10 µl of 10X CIAP buffer (Promega, UK), 3 pmol ends of DNA probe, 2 µl of CIAP (Promega, UK) and dH_2O to a total volume of 100 µl. The reaction was incubated at 37 °C for 30 minutes, a further 1 µl of CIAP (Promega, UK) was added and the incubation continued for a further 30 minutes. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA and the sample was heated at 65 °C to inactivate the

CIAP. The DNA probe was extracted by the addition of 90 µl of phenol/chloroform/isoamyl alcohol (25:24:1), the sample was vortexed and spun at full speed for 5 minutes and the upper aqueous layer was transferred to a fresh tube. The sample was then ethanol precipitated (Section 2.2.11), air dried and dissolved in 20 µl dH_2O.

2.4.9.3 Labeling (^{32}P) and digestion of probe DNA

The DNA probe (Sections 2.4.9.1-2.4.9.2) was radiolabeled with [γ-^{32}P]ATP using T4 PNK in the following reaction: 10 µl DNA probe (3 pmol ends of 0.5 kb promoter DNA), 2 µl 10X kinase buffer (Promega, UK), 1 µl T4 PNK (Promega, UK), 1 µl [γ-^{32}P]ATP (3,000Ci/mmol, 10mCi/ml, Amersham, UK) and dH_2O to a total volume of 20 µl. The reaction was incubated at 37 °C for 30 minutes, an additional 1 µl of T4 PNK was added and the incubation was continued for a further 30 minutes. The labeled DNA probe was then digested for 1 hour at 37 °C with *Xba*I (Section 2.2.1) to remove the 3' end ^{32}P-label, leaving a 526 bp fragment. The digested sample was then phenol extracted (Section 2.4.9.2) and ethanol-precipitated (Section 2.2.11). The DNA probe was air dried, dissolved in 100 µl of TE (pH 7.4) and stored at 4° C. The radiolabeled probe was then counted in a 1.5 ml microcentrifuge tube using a Beta Scout 2007 liquid scintillation tester (Perkin-Elmer, USA). Specific activity of the 0.5 kb promoter fragment was between 1 x 10^6 and 2 x 10^6 cpm/pmol of DNA.

2.4.9.4 DNase I Footprinting reaction

Two separate reactions were set up for a basic footprint, Reaction 1 contained only the DNA probe and was used to obtain an unprotected DNA ladder. Reaction 2 contained all the components of Reaction 1 along with the nuclear extract. The two reactions were treated identically throughout. Reactions were prepared as follows:

Reaction 1 (Negative control)
20µl Nuclease free water
25µl Binding buffer (Promega, UK)
5 µl DNA probe (0.15 pmol/ 80,000 cpm)
0 µl Nuclear extract (NE)
50 µl Total volume

Reaction 2 (Positive control)
18 µl Nuclease free water
25 µl Binding buffer (Promega, UK)
5 µl DNA probe (0.15 pmol/ 80,000 cpm)
2 µl NE
50 µl Total volume

The reactions were mixed and incubated on ice for 10 minutes. To each reaction 50 µl room temperature Ca^{2+}/Mg^{2+} solution (Promega, UK) was added, samples were mixed and incubated at room temperature for one minute. Three µl (0.15 units) of diluted RQ1 RNase-Free DNase I (Promega, UK) was added, and the reactions were mixed and incubated at room temperature for exactly one minute. The reactions were immediately terminated by the addition of 90 µl of 37 °C stop solution (Promega, UK). The samples were phenol extracted (Section 2.4.9.2), ethanol precipitated (Section 2.2.11) and air-dried, before being dissolved in 10 µl Loading Buffer (Promega, UK). Finally, the samples were denatured at 95° C for 2 minutes, chilled on ice and 5 µl was loaded onto a 4 % sequencing gel (Section 2.3.4).

2.5 General methods for cell culture

2.5.1 Ovine immortalised cell cultures – sA80BR and pA80BR

Immortalised ovine cell cultures, derived from brain tissue from NPU Cheviot sheep of the VRQ/VRQ genotype (scrapie susceptible sheep – sA80BR) and the ARQ/ARR genotype (scrapie resistant sheep – pA80BR), were available at the NPU (Figure 2.1). Cell cultures were produced from NPU Cheviot sheep by Dr Huw John, Moredun Research Institute, Edinburgh (John, 1994, Marshall, 2000).

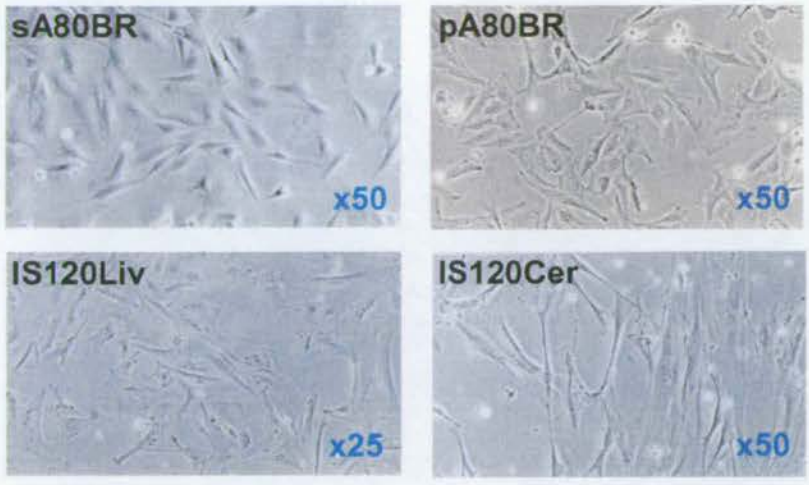


Figure 2.1 *Ovine neuronal and peripheral cell cultures*

Photographs of the cell lines were kindly provided by Dr. Gerry O'Neill, NPU. sA80BR = sheep neuronal cell line derived from scrapie susceptible sheep of genotype VRQ/VRQ. pA80BR = sheep neuronal cell line derived from scrapie resistant sheep of genotype ARQ/ARR. IS120Liv = Liver cell line, IS120Cer = Cerebellum cell line, derived from an Icelandic sheep of the genotype ARQ/ARQ shown to be susceptible to scrapie in Icelandic sheep. Magnifications are given in the bottom right corner of each photograph.

2.5.2 Ovine primary cell cultures – IS120Cer, IS120Liv, IS120Med & IS120Kid

A series of primary ovine cell cultures were produced by Dr Gerry O'Neill from Icelandic sheep tissue (*Ovis brachyura borealis pall*). The sheep used was one and half years old and its scrapie genotype was ARQ/ARQ, shown to be scrapie susceptible in Icelandic sheep. Cell cultures included a cerebellum derived cell culture (IS120Cer), a liver derived cell culture (IS120Liv), a kidney derived cell culture (IS120Kid) and a brain medulla derived cell culture (IS120Med) (IS120Kid not shown, Figure 2.1).

2.5.3 Characterisation of the ovine brain derived cell cultures

Marshall (2000) characterised the ovine brain derived cell cultures, sA80BR, pA80BR and IS120Cer. Antibodies specific for astrocytes/glia (anti-glial fibrillary

acidic protein (GFAP, Sigma G-3893)), neurons (anti-neurofilament 200 (N200, Sigma N-0142)) and fibroblasts (anti-fibronectin (Sigma F-3648)) were used to characterise the cell types present in the ovine brain cell cultures. These experiments indicated that the immortalised cell cultures, sA80BR and pA80BR, contained glia/astrocytes and neurones and were free of fibroblast cells. The primary Icelandic cell culture IS120Cer reacted positively for neuronal cells, and negatively for glia/astrocytes and fibroblast cells.

2.5.4 Culturing of ovine neuronal cell lines sA80BR and pA80BR

Cells were grown in Nerve growth factor (NGF) complete medium (NCM) which consisted of 500 ml Dulbecco's Modified Eagle's Medium (MEM) + Glutamax (Invitrogen, UK), 0.5 ml NGF (Murine 7-s-NGF, Sigma, UK, 10 ng/ml), 50 ml fetal calf serum (Globeparm, UK) and 5 ml penicillin/streptomycin (Invitrogen, UK, 100 U/ml). Cells used for the studies in this thesis were of low passage number (between passage number 10-20). Cells were incubated at 33 °C (due to being immortalized with a temperature-sensitive form of SV40 Large T antigen) with 5 % CO_2 in 75 cm^2 flasks (Corning, UK) until confluency was reached. Cultured cells were fed every 2-3 days with fresh NCM.

2.5.5 Culturing of murine neuroblastoma cell line N2a and Icelandic sheep primary cell lines

The cell growth requirements of the N2a and the Icelandic sheep primary cell lines differ from those of the ovine neuronal cell lines (sA80BR and pA80BR, Section 2.5.1). These cells were incubated at 37 °C with 5 % CO_2 in 75 cm^2 flasks and cultured in standard complete medium (SCM) which has the same composition as NCM with the exception of NGF. Icelandic sheep cell lines were of low passage number (between passage number 10-20) and the murine N2a cells were of passage number 100-200. Cultured cells were fed every 2-3 days with fresh SCM.

2.5.6 Handling cells for passage

Culture medium was removed and the cells were rinsed twice with pre-warmed (37 °C) sterile PBS (Invitrogen, UK). Five hundred µl trypsin/versene solution (Invitrogen, UK) was added and the flask was rocked gently for 1 minute, or until all the cells had detached from the base of the flask. Ten ml of fresh culture medium was added to the cells to inactivate the trypsin. An aliquot of the trypsinised cells was transferred to a fresh flask along with 8 ml of fresh culture medium and the cells were cultured as described previously (Sections 2.5.1 - 2.5.2).

2.5.7 Calculating cell counts using a haemocytometer

Cells were harvested (Section 2.5.3) and re-suspended in 2 ml of the appropriate culture medium (Sections 2.5.1 – 2.5.2). The haemocytometer slide and coverslip were cleaned with 70 % ethanol. The coverslip was lightly moistened and placed on top of the haemocytometer before pressing the coverslip into position. An aliquot of the cell suspension was transferred to the haemocytometer using a glass Pasteur pipette (Bilbate, UK) and the cells were allowed to settle before counting began. Cells were counted in 10 primary squares using the following rules:

1. If too many cells were present to count, a 1:2 dilution in PBS was performed.
2. The middle of the triple lines separating each primary square was regarded as the boundary. Cells that touched the upper or left boundaries were included, those that touched the bottom or right boundaries were excluded.
3. When the haemocytometer was loaded, the volume of the cell suspension that occupied one primary square was 0.1 mm^3 (1.0 mm^2 x 0.1 mm) or 1.0 $x10^{-4}$ ml.
4. Counting 10 primary squares gave the number of cells within 1.0 mm^3 (10 x 0.1 mm^3) or 1 x 10^{-3} ml. Total cell concentration in the original suspension (cells/ml) was calculated by the following equation:

Cells/ml = total count x 1000 x dilution factor

2.5.8 Preparing cells for PrP^c protein analysis

Cultured cells for PrP^c protein analysis were cultured in six well plates (Corning, UK). Following the inactivation of trypsin during passage (Section 2.5.3) 500 µl of cell suspension was transferred to each well of a six well plate. A further 1.5 ml of fresh culture medium was added and cells were cultured as described previously for 48 hours or until confluency was reached (Sections 2.5.1 – 2.5.2).

2.5.9 Preparation of cell lysates from cultured cells and tissue samples

Three different methods of cell lysate production were utilised throughout this thesis. The protocols for these methods are described below. In all cases the culture medium was removed from the cells cultured in six well plates (Section 2.5.5) and cells were washed once in 2 ml ice cold PBS.

2.5.9.1 Preparation of murine tissue for protein extraction

Whole mouse brain samples were homogenised in 250 µl ice cold PBS. An aliquot of 75 µl was removed from this homogenate and further diluted in 500 µl ice cold PBS, cells were then isolated by centrifugation at 1,000 g for 10 minutes. Protein extracts were produced using either the RCLB, M-per or Mem-per methods described below.

2.5.9.2 Reporter cell lysis buffer (RCLB) method

A sufficient volume of 1X RCLB (Promega, UK) was added to cover the cells (~ 400 µl for a single well of a six well plate) along with 50 µl Complete Mini - proteinase inhibitor solution. The plate was rocked gently for 15 minutes at room temperature. The wells were scraped using a sterile cell scraper (Corning, UK), ensuring that all visible cell debris was collected and using a pipette the cell lysate was transferred to a pre-chilled 1.5 ml microcentrifuge tube and placed on ice for no more than two minutes prior to centrifugation. The cell lysate was cleared of cell debris by centrifugation at 1,000 x g for 10 minutes and the supernatant containing

the protein fraction (~500 µl) was transferred to a fresh, pre-chilled microcentrifuge tube ready for immunoprecipitation (Section 2.7.3).

2.5.9.3 Mammalian protein extraction reagent (M-per) method

To each well, 400 µl of M-per (Perbio, UK) and 50 µl Complete Mini - proteinase inhibitor solution were added drop-wise onto the cells and the plate was shaken gently at room temperature for 5 minutes. The cell lysate (~ 500 µl per well) was transferred to a pre-chilled 1.7 ml microcentrifuge tube (Corning, UK) and spun at 1,000 x g for 10 minutes at 4 °C to remove cell debris. The supernatant containing the protein fraction (~ 500 µl) was transferred to a fresh pre-chilled microcentrifuge tube ready for immunoprecipitation (Section 2.7.3).

2.5.9.4 Mammalian membrane protein extraction reagent (Mem-per) method

To each well, 500 µl of sterile PBS (Invitrogen, UK) was added and the cells were scraped using a sterile cell scraper (Corning, UK). Scraped cells were transferred to a pre-chilled 1.7 ml microcentrifuge tube and spun at 3,000 x g for 2 minutes in a pre-chilled rotor. The supernatant was discarded and the cell pellet was re-suspended in 150 µl of Mem-per Reagent A (Pierce, UK) and incubated at room temperature for 10 minutes with occasional vortexing. During this incubation 50 µl of Complete Mini - proteinase inhibitor solution was added and the solution was placed on ice. Mem-per Reagent C was diluted in a ratio of 2:1 with Mem-per Reagent B and placed on ice. Reagent C (450 µl) was added to the cell lysate and placed on ice for 30 minutes with vortexing every 5 minutes. The tube was spun at 10,000 x g for 3 minutes at 4 °C and the supernatant was transferred to a fresh tube. The supernatant was incubated at 37 °C for 10 minutes to separate the membrane protein fraction. The tube was spun at 10,000 x g for 2 minutes at room temperature to isolate the hydrophobic fraction. The bottom layer containing the membrane proteins (~ 250 µl) was then transferred to a fresh pre-chilled microcentrifuge tube ready for immunoprecipitation (Section 2.7.3).

2.5.10 Determination of total protein concentration in cell lysates – Bio-Rad protein assay II

Total protein concentration of cell extracts (Section 2.5.6) was determined using the Bio-Rad protein assay II, following the manufacturer's protocol. A protein standard curve was produced by diluting the protein standard (1.4 mg/ml) as follows:

1.4 mg/ml = Standard
1.05 mg/ml = 25 µl dH_2O + 75 µl standard
0.7 mg/ml = 50 µl dH_2O + 50 µl standard
0.35 mg/ml = 50 µl dH_2O + 50 µl (0.7 mg/ml solution)
0 mg/ml = 100 µl dH_2O

A protein standard curve was prepared as follows:

1 = Standard (1.4 mg/ml)
2 = Standard (1.05 mg/ml)
3 = Standard (0.7 mg/ml)
4 = Standard (0.35 mg/ml)
5 = Blank (0 mg/ml)

The protein assay was performed, according to the manufacturer's instructions, briefly, 100 µl of the appropriate standard (or dH_2O for the blank sample) and 5 ml diluted dye reagent was added to each tube. Samples were incubated at room temperature for 1 hour, 1 ml aliquots were removed and the absorbance at 595 nm was read on a spectrophotometer. A standard curve was plotted of absorbance against protein concentration and the equation of the curve was determined using the Microsoft Excel package (Microsoft, USA). The reaction detailed above was then repeated for the protein samples (cell extracts) and total protein concentration present in the extracts was determined from the absorbance value at 595 nm using the equation of the standard curve.

2.5.11 Total RNA extraction from cultured cells with RNAzol reagent B

Total RNA was extracted from cultured cells using the RNAzol B reagent (AMS Biotechnology, UK). Cells were cultured in six well plates (Section 2.5.5), culture medium was removed and the wells were washed twice in 2 ml ice-cold PBS.

One ml of RNAzol B reagent was added to each well, the cells were disrupted by gentle rocking and the RNA was solubilised by pipetting. The RNA solution was transferred to a pre-chilled 1.5 ml microcentrifuge tube, 0.2 ml chloroform (BDH, UK) was added, the solution was mixed by vortexing and incubated on ice for 15 minutes. The sample was centrifuged at 12,000 x g for 15 minutes at 4 °C and the upper aqueous layer was transferred to a fresh pre-chilled microcentrifuge tube. The RNA was precipitated in an equal volume of iso-propanol and incubated for 5-10 minutes at room temperature. The sample was spun at 12,000 x g for 5 minutes at 4 °C and the supernatant was discarded. The RNA pellet was washed in 1 ml of 75 % ethanol, spun as before, air dried, re-suspended in 75 % ethanol and stored at –20 °C until required.

2.5.12 Freezing cells for long term storage

Trypsinised cells (Section 2.5.3) were collected into a 15 ml plastic centrifuge tube, centrifuged at 1,000 x g for 2 minutes and the supernatant discarded. The cell pellet was re-suspended in 3 ml freezing down medium and 1 ml aliquots of the re-suspended cells were transferred to 1.8 ml cryovials (Corning, UK). The cryovials were then wrapped in tissue paper and stored overnight at – 70 °C. The following day the cryovials were transferred into liquid N_2 until required.

2.5.13 Thawing cells from liquid nitrogen storage

The cryovial containing the frozen cells (Section 2.5.9) was removed from liquid N_2 and the cells were thawed slowly in a water bath at 37 °C. To prevent contamination of the contents the outside of the cryovial was sprayed with 70 % ethanol. The contents of the cryovial were transferred to a clean 15 ml plastic centrifuge tube and 10 ml of culture medium was added to dilute out the dimethyl sulphoxide (DMSO). The cells were pelleted at 1,000 x g for 2 minutes and the supernatant was discarded. The cell pellet was re-suspended in 10 ml of fresh culture medium and transferred to a 75 cm^2 flask. The cells were then cultured as described previously (Sections 2.5.1 - 2.5.2).

2.5.14 Testing cell cultures for presence of mycoplasma

All cell cultures were tested for the presence of mycoplasma contamination on a three-monthly basis and always found to be negative. This test was carried out using the Mycoplasma *Plus* PCR primer set (Stratagene, UK) following the manufacturer's instructions.

2.6 General methods for transient transfection

2.6.1 Preparation of DNA for transfection

DNA used for transfections was purified using CsCl gradients (Section 2.2.12). For transfections an optical density (OD) ratio at OD_{260}:OD_{280} of approximately 1.9 indicating a good level of purity was used, with a final DNA concentration of approximately 1 mg/ml (Section 2.2.13).

2.6.2 Control vector – pSV-β-galactosidase

The control vector pSV-β-galactosidase (Promega, UK) was used to optimise transfection conditions and as a control for transfection efficiency by analysing reporter gene activity in transfected cell lysates. It was assumed that both the reporter vector and the transfected DNA would be transfected with equal efficiency in a single reaction, but that different cell populations would be transfected at varying efficiencies. Therefore measuring the amount of β-galactosidase activity in the transfected cell extracts allowed the level of efficiency to be determined for individual cell populations. The volume of each cell lysate used for protein analysis was then adjusted according to the efficiency of the transfection reaction (Section 4.2.3)

2.6.3 Beta-galactosidase enzyme assay

The β-galactosidase enzyme assay system (Promega, UK) was used to measure β-galactosidase activity in the transfected cell lysates. A 250 µl aliquot of the cell extract was added to 250 µl of 2X assay buffer, mixed and incubated at 37 °C

for 30 minutes. The reaction was stopped by the addition of 500 µl of 1M $NaCO_3$ and the colour reaction was measured by reading the absorbance at 420 nm. The absorbance value at 420 nm was compared to a standard curve (Section 2.6.4) prepared using a β-galactosidase standard (Promega, UK), to determine the concentration of β-galactosidase in the cellular extract (Section 2.6.6).

2.6.4 Preparation of a β-galactosidase standard curve

A β-Galactosidase standard curve was prepared using a β-galactosidase standard (1 U/µl, Promega, UK). The standard was diluted in the appropriate cell lysis reagent i.e. M-per (Pierce, UK), Mem-per (Pierce, UK), or RCLB (Promega, UK). Ten µl of β-galactosidase standard (1 U/µl) was diluted in 990 µl of the appropriate lysis buffer and mixed. Ten µl of this 1:100 dilution was further diluted in 990 µl of lysis buffer and mixed to create a 1:10,000 stock solution. A range of dilutions was prepared between 0 and 6 milli-units of β-galactosidase using the 1:10,000 stock solution as detailed below (Table 2.4).

Dilution	β-galactosidase (milli-units)	1:10,000 stock (µl)	Cell lysis reagent (µl)
1	0	0	150
2	1	10	140
3	2	20	130
4	3	30	120
5	4	40	110
6	5	50	100
7	6	60	90

Table 2.4 *β-galactosidase standard curve dilutions*

Table details the preparation of the dilutions of the *β-galactosidase* enzyme for the standard curve used to determine the level of *β-galactosidase* activity in cell lysates following transient co-transfection with *β-galactosidase* DNA and ovine PrP mini-gene DNA. *β-galactosidase* stock solution was diluted with the above detailed volume of the appropriate cell lysis reagent (M-per or Mem-per) to produce the standard solutions used for the standard curve.

The standards were processed using the β-galactosidase assay (Section 2.6.3) and the absorbance values at 420 nm were determined for each standard using a spectrophotometer. Using these values a standard curve was plotted of protein concentration against absorbance and the equation of the line was determined using the Microsoft Excel package (Microsoft, USA).

2.6.5 Dendrimer technology – Superfect

Superfect reagent (Qiagen, UK) was chosen as the transfection agent for the ovine neuronal cell lines as previous work had shown that it was the most efficient method of transfecting the ovine cell lines (Marshall, 2000). Superfect consists of activated dendrimer molecules with a defined spherical shape. Branches terminating in charged amino groups radiate out from a central core and these are able to interact with the negatively charged phosphate groups of nucleic acids. The DNA is packaged into compact structures of uniform size and defined shape, therefore optimising the entry of DNA into the cell.

2.6.6 Superfect transfection reagent protocol

Cells were transfected with Superfect transfection reagent according to the manufacturer's instructions. Cells were split into six well plates 2-3 days prior to transfection to ensure that they would be 60-80 % confluent on the day of transfection (Section 2.5.5). CsCl prepared plasmid DNA (Section 2.2.12) was diluted in TE buffer (pH 7.4) to the optimum concentration required for the cell line to be transfected (Section 2.6.7). To the diluted DNA, 100 µl growth medium containing no serum or antibiotics was added. The appropriate amount of Superfect reagent (Qiagen, UK) for the cell line used was added (Section 2.6.7), the solution was mixed and incubated at room temperature for 10 minutes to allow DNA-dendrimer complexes to form. While the complexes were forming, the growth medium was removed from the cells in each well, the cells were washed twice with 1 ml pre-warmed PBS (37 °C). At this stage 600 µl of growth medium, containing serum and antibiotics, was added to the DNA-dendrimer complex solution. The solutions were mixed by pipetting and immediately added drop-wise to the cells in

each well. The cells were incubated as normal for 3 hours (Sections 2.5.1 - 2.5.2), after which time the remaining complexes were removed and fresh culture medium (containing serum and antibiotics) was added. The cells were then incubated as normal (Sections 2.5.1 - 2.5.2) until harvested for protein analysis (Section 2.5.3), between 24-72 hours later.

2.6.7 Optimisation of transfection conditions for individual cell lines

Superfect transfection conditions for the ovine cell lines were optimised using the following protocol and utilising the β-galactosidase assay (Section 2.6.3). Cells were cultured as described in Section 2.6.6. DNA mastermixes (A, B & C) were prepared as described below:

A = 4 μg β-galactosidase DNA in 400 μl culture medium (no serum/antibiotics)
B = 8 μg β-galactosidase DNA in 400 μl culture medium (no serum/antibiotics)
C = 16 μg β-galactosidase DNA in 400 μl culture medium (no serum/antibiotics)

The DNA mastermixes were mixed by vortexing, incubated at room temperature for 5 minutes and centrifuged briefly at full speed. Eleven 0.5 ml microcentrifuge tubes were labelled (1-11) and pre-chilled on ice. One hundred μl of DNA mastermix A were placed into tubes 1-3, 100 μl of DNA mastermix B were placed into tubes 4-6 and 100 μl of DNA mastermix C were placed into tubes 7-9. As controls 100 μl growth media (no serum/antibiotics) were placed into tubes 10 & 11. Superfect reagent was added to each tube as detailed in Table 2.5, the tubes were mixed by vortexing and incubated at room temperature for 10 minutes.

Tube number	Superfect added (µl)
1	2
2	5
3	10
4	4
5	10
6	20
7	8
8	20
9	40
10	10
11	0

Table 2.5 *Quantities of Superfect added to each cell culture transfection reaction*

Shows the quantity (µl) of Superfect reagent added into each cell culture transfection reaction for the optimisation of the transfection conditions as used for each individual cell line.

During this incubation the culture medium was removed from the cells and they were washed once in 2 ml ice cold PBS. Six hundred µl of culture medium (containing serum and antibiotics) was added to each of the tubes (numbered 1-11), the contents of each of the eleven tubes were then added drop-wise to eleven of the twelve wells of the two six well plates. To the twelfth well 1 ml of culture medium (containing serum and antibiotics) was added. The six well plates were swirled gently to evenly distribute the culture medium and the cells were incubated as previously described (Sections 2.5.1 - 2.5.2) for 3 hours. The media was removed, the cells were washed in 2 ml ice cold PBS, 2 ml fresh culture medium (containing serum and antibiotics) was added and the cells were cultured as previously described (Sections 2.5.1 - 2.5.2) for a further 24 hours. Cells were harvested (Section 2.5.3), lysed (Section 2.5.6) and cell lysates were then analysed for β-galactosidase activity using the β-galactosidase assay (Section 2.6.3). The optimum amount of transfected DNA and Superfect to be added for each individual cell line was then determined.

2.7 General methods for isolation of PrP^c from cell lysates

2.7.1 Methanol precipitation

For isolation of total protein from a 500 µl cell lysate (Section 2.5.6), nine times the sample volume of pre-chilled (–20 °C) methanol was added, the sample was mixed and incubated at –20 °C for at least one hour. Precipitated proteins were centrifuged at 1,000 x g for 10 minutes. The pellet was re-suspended in 30 µl of NuPage sample buffer (Invitrogen, UK), 3 µl sample reducing agent (Invitrogen, UK) was added and the sample was denatured at 70 °C for 10 minutes prior to electrophoresis (Section 2.8.1).

2.7.2 Chloroform/methanol precipitation

For isolation of total protein from a 500 µl cell lysate (Section 2.5.6), 1.6 ml methanol, 0.8 ml chloroform and 1.2 ml dH_2O was added to the 500 µl cell lysate in a 15 ml centrifuge tube (Corning, UK). The preparation was vortexed and spun at 1,000 x g for 10 minutes. Pellets were re-suspended in 30 µl of NuPage sample buffer, 3 µl sample reducing agent (Invitrogen, UK) was added and the sample was denatured at 70 °C for 10 minutes prior to electrophoresis (Section 2.8.1).

2.7.3 Immunoprecipitation of PrP^c from cell lysates

For the isolation of PrP^c from cell lysates the following immunoprecipitation method based on that described by Firestone & Winguth (1990) was developed. For isolation of PrP^c from a 500 µl cell lysate (Section 2.5.6), 1 µl of the appropriate precipitating antibody was added (Table 2.6) and incubated at 4 °C for 1 hour with end over end shaking on a SB1 blood tube rotator (Stuart Scientific, UK). To the same tube 50 µl bed volume of protein G sepharose (stored in PBS at 4 °C, Pharmacia Biotech, SE) was added and incubated at 4 °C with overnight with shaking to allow the formation of immuno-complexes. The immuno-complexes were collected by centrifugation for 25 seconds at full speed and the supernatant was discarded. The sample was re-suspended in 1 ml immunoprecipitation (IP) buffer 1

(0.05 M tris, 0.15 M NaCl, 1 % nonidet P-40) and incubated for 20 minutes at 4 °C with end over end shaking. Immuno-complexes were again collected by centrifugation and the sample was re-suspended in 1 ml fresh IP buffer 1 and incubated as before. This procedure was repeated a further two times using IP buffer 2 (0.05 M tris, 0.5 M NaCl, 1 % nonidet P-40) and then once using IP buffer 3 (0.05M tris). The beads were collected as before and any last traces of the final wash from the walls and lid of the tube were blotted dry with tissue. The pellet was re-suspended in 30 µl of sample buffer and 3 µl of sample reducing agent was added. Samples were denatured by heating to 70 °C for 10 minutes, protein A sepharose was removed by centrifugation at full speed for 1 minute and the supernatant was analysed by electrophoresis (Section 2.8.1).

2.7.4 Precipitating antibodies

The PrP specific polyclonal antibodies 1A8 and 1B3 (Farquhar, Somerville & Ritchie, 1989) were used as the major antibodies for immunoprecipitation studies. In addition to these a number of monoclonal antibodies (Jacquie Manser & Dr. Sandra McCutcheon, IAH Compton, unpublished) raised against both full length and truncated PrP^c were analysed for their ability to precipitate PrP^c from a variety of cell lines (Table 2.6). In addition the anti-FLAG™ monoclonal antibody M2 and an anti-FLAG™ polyclonal antibody were used to immunoprecipitate 3XFLAG™ tagged recombinant PrP (Table 2.6).

Antibody	Epitope(s)	Specificity	Concentration and subclass	Supplier
1A8	Multiple epitopes on PrP	Sheep, goat, mouse, hamster, cattle, pig, cat and human	1 µg/µl, Rabbit, IgG	Christine Farquhar (NPU)
1B3	Multiple epitopes on PrP	Sheep, goat, mouse, hamster, cattle, pig, cat and human	1 µg/µl, Rabbit, IgG	Christine Farquhar (NPU)
AB1	PrP C-terminal region	Sheep, hamster, cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
AB2	PrP C-terminal region	Sheep, hamster, cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
AB3	PrP C-terminal region	Sheep, hamster, cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
AB4	PrP C-terminal region	Sheep and cattle	2 µg/µl	S. McCutcheon (IAH-Compton)
AB5	PrP C-terminal region	Sheep and cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
AB6	PrP C-terminal region	Sheep, hamster, cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
AB7	PrP C-terminal region	Sheep, hamster, cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
AB8	PrP C-terminal region	Sheep, hamster, cattle, human and mouse	2 µg/µl	S. McCutcheon (IAH-Compton)
BE12	PrP N-terminal region	Human, hamster, cattle, sheep, mouse	1.3 µg/µl, IgG1	J. Manser (IAH-Compton)
AH6	PrP N-terminal region	Human, hamster, cattle, sheep, mouse	1.4 µg/µl, IgG2a	J. Manser (IAH-Compton)
AG4	PrP N-terminal region	Human, hamster, cattle, sheep, mouse	1.3 µg/µl, IgG2b	J. Manser (IAH-Compton)
M2 monoclonal	FLAG™ sequence DYKDDDDK	FLAG™ sequence at any location on recombinant protein	2-5 µg/µl, IgG1	Sigma-Aldrich (Cat. No. F3165), UK
α-FLAG polyclonal	FLAG™ sequence DYKDDDDK	FLAG™ sequence at any location on recombinant protein	1 µg/µl Rabbit, IgG	Sigma-Aldrich (Cat. No. F7425), UK

Table 2.6 *Antibodies used to immunoprecipitate PrP*

Details the antibodies used for the immunoprecipitation of endogenous and recombinant (3XFLAG tagged) PrP from cell cultures. Epitope, specificity, concentration and subclass of the antibodies is given where appropriate and when known, as is the source of the antibody.

2.8 General methods for analysis of PrP^c isolated from cell lysates

2.8.1 SDS-PAGE and Western blot analysis of PrP^c isolated from cell culture extracts

The following protocols were used as first described by Laemmli (1970) and Towbin, Staehelin & Gordon (1979). Denatured, immunoprecipitated cell culture extracts (Section 2.7.3) were loaded onto a pre-cast 4-12 % bis-tris gel (Invitrogen, UK). In addition 5 µl of molecular weight marker (Invitrogen, UK) was loaded on the gel to give an approximation of band size, and 0.01g of scrapie positive sheep brain sample was also loaded as a PrP^{sc} positive control (Angie Chong, NPU). The gel was run for 50 minutes at 200 V constant/110-125 mA on an XCell Surelock Gel System (Invitrogen, UK). Whilst the gel was running two (6 x 8.5 cm) pieces of 1F filter paper (Munktell, SE) were cut and soaked in transfer buffer (Invitrogen, UK) along with 5 foam blotting pads (Invitrogen, UK). One piece (6 x 8.5 cm) of Immobilon-P PVDF transfer membrane (Millipore, UK) was soaked in methanol for 5-10 seconds, washed in distilled water for 2 minutes and soaked in transfer buffer for 5 minutes. Once the gel run was completed the gel cassette was disassembled and the foot and wells of the gel were removed with a gel knife. A piece of pre-soaked filter paper was placed on top of the gel, the gel was turned over and the pre-soaked PVDF was placed on top of the gel and a further piece of filter paper was then placed on top of the PVDF. The gel sandwich was carefully placed into the XCell II blot module (Invitrogen, UK), sandwiched on either side by two pre-soaked blotting pads and a further blotting pad was added to ensure a tight fit. The XCell II blot module was placed into the XCell Surelock cell and filled with 250 ml of transfer buffer (Invitrogen, UK), the outer chamber was filled with 800 ml dH_2O and protein samples were transferred to the PVDF membrane at 30 V constant /170 mA for 1-1.5 hours. The gel sandwich was disassembled carefully and the PVDF blot was removed with clean tweezers, washed in methanol for 5 seconds and left to dry in a fume cabinet for 10-15 minutes at room temperature on a piece of Whatman 3MM filter paper. The blot was re-wetted with methanol, washed with water and washed twice in TBS. The blot was blocked with 25 ml of 1 % blocking solution

(Roche, UK) at room temperature for 1 hour with shaking on a Belly Dancer (Stovall Life Sciences, USA). The primary antibody 6H4 (Section 2.8.2), diluted in 1 % blocking solution (1:10,000 dilution, Prionics, CH) was added and incubated overnight at room temperature with shaking. The blot was washed twice for 10 minutes with 25 ml of 0.5 % blocking solution, then the secondary antibody (Section 2.8.3), diluted in 0.5 % blocking solution (1:25,000 dilution, Jackson Immuno-research Lab Inc, USA) was added and incubated at room temperature for 60 minutes with shaking. The blot was washed for 1 hour with four changes of TBST (20 ml) and finally rinsed with water for 90 seconds. The blot was overlaid onto pre-mixed detection reagent (Roche, UK), incubated at room temperature for 60 seconds, excess detection reagent was drained and the blot was placed between two pieces of 3M transparent film (Fisher, UK). The blot was exposed to Kodak XAR-5 film (Roche, UK) for 30 seconds, 3 minutes and 10 minutes respectively.

2.8.2 Primary antibodies

At the time of writing no antibody was available that could reliably distinguish between PrP^{c} and PrP^{sc}. In addition, most antibodies will cross-react with PrP protein from two or more species, due to the high level of conservation between PrP molecules of different species. The commercially available monoclonal antibody 6H4 (Prionics, CH) was used as the major primary antibody for chemiluminescence blotting throughout this thesis. This antibody shows good specificity to PrP from humans, cattle, sheep, mouse, hamster, mink and a variety of primates (Table 2.7). In addition the FLAG specific M2 antibody was used to detect 3XFLAG™ tagged recombinant PrP (Table 2.7).

Antibody	Epitope (s)	Specificity	Concentration and subclass	Supplier
M2 monoclonal	Flag™ sequence DYKDDDDK	FLAG™ sequence at any location in recombinant protein	2-5 µg/µl, IgG1	Sigma-Aldrich, UK (Cat. No. F3165)
6H4	DYEDRYYRE	Human, cattle, sheep, rabbit, mink, mouse, hamster, rat and a variety of primates	1 µg/µl, IgG1	Prionics, Switzerland (Cat. No. 01-010)

Table 2.7 *Primary antibodies*

Details the primary antibodies used for the Western blot detection of endogenous and recombinant (3XFLAG tagged) PrP from cell cultures. Epitope, specificity, concentration and subclass of the antibodies are given along with the source.

2.8.3 Secondary antibody

The secondary antibody used in these studies was a peroxidase conjugated affinipure rabbit anti-mouse IgG (Stratech, UK). This antibody was used for the detection of primary antibody bound to PrP following chemiluminescence blotting (Table 2.8).

Antibody	Epitope (s)	Specificity	Concentration	Supplier
Peroxidase conjugated affinipure rabbit anti-mouse IgG	Heavy chains on mouse IgG and light chains on most mouse immunoglobulins.	Mouse but may also react with other species.	0.8 µg/µl	Jackson Immuno-Research Laboratories, USA

Table 2.8 *Secondary antibody*

Details the secondary antibody used for the Western blot detection of endogenous and recombinant (3XFLAG tagged) PrP from cell cultures. Epitope, specificity and concentration is given along with the source.

2.8.4 Optimisation of primary and secondary antibody blotting conditions using dot blots

In order to determine the optimum dilution of primary antibody used for Western blotting, six single strips (6 cm x 4 cm) of nitrocellulose membrane (Schleicher & Schuell, DE) were prepared, and each strip was divided into six sections with a pencil. All of the strips were soaked in TBS for 2 minutes, then kept moist by placing them onto a pre-soaked bed of filter paper (3MM, Whatman, UK). Six serial dilutions of the antigen (3XFLAG™-BAP fusion protein, Sigma-Aldrich, UK) were produced in TBS (1 μg to 10 pg). A 1μl aliquot of each antigen dilution was carefully spotted onto the divided nitrocellulose membrane, and this was repeated for all six nitrocellulose strips, which were allowed to dry at room temperature for 5 minutes. Each strip was blocked for 1 hour in 1 % blocking solution at room temperature. Five serial dilutions of the primary antibody (M2 anti-FLAG™, Sigma-Aldrich, UK) were produced (1:500, 1:1,000, 1:2,000, 1:5,000, 1:10,000) in 1 % blocking solution. One strip was not incubated in primary antibody, instead it was incubated in TBST to provide a background control. The remaining five strips were each incubated in one of the primary antibody dilutions at room temperature for 2 hours, with constant shaking. All six strips were washed briefly three times in TBST, then incubated with the secondary antibody (Section 2.8.3; 1:20,000) at room temperature for 1 hour. The strips were washed five times in TBST, and samples were detected as described in Section 2.8.1.

In order to determine the optimum dilution of the secondary antibody, the procedure described above was repeated, however, the optimum primary antibody dilution determined above was used for all six strips. Five serial dilutions of the secondary antibody (Section 2.8.3; 1:50,000, 1:40,000, 1:25,000, 1:20,000, 1:10,000) were produced and five of the six strips were each incubated in one of the secondary antibody dilutions, the remaining strip was not incubated in secondary antibody, instead it was incubated in TBST to provide a background control. Samples were then detected as described in Section 2.8.1.

Chapter 3: Development of techniques for the extraction and analysis of PrP^c from cell culture extracts and tissue samples

3.1 Introduction

The detection of PrP mRNA has been used to determine the level of PrP gene expression, however, it may not give an accurate picture of the cellular level of PrP^c (Caughey *et al.*, 1988). For example, Gygi, Rochon, Franza *et al.* (1999) showed that for certain genes in yeast, mRNA and protein levels varied by up to 20-fold. Furthermore, Ford *et al.* (2002) showed that although the level of PrP^c expression varies between different cell-types in the brain, the level of PrP mRNA remains high, even in neurones which showed no detectable PrP^c. The authors concluded that the steady state level of PrP^c is primarily controlled in a post transcriptional manner, and that this is probably regulated by differences in protein trafficking or degradation. As such, the cellular level of PrP mRNA could be high in comparison to the level of the PrP^c protein because of the relatively short half-life of PrP^c (3-6 hours) and the detection of PrP mRNA may not necessarily reflect the actual level of PrP^c protein (Borchelt *et al.*, 1990, Caughey, 1993, Pfeifer, Bachmann, Schroder *et al.*, 1993). Therefore, the determination of PrP gene activity via mRNA analysis alone may not be sufficient. In order to obtain a more accurate picture of ovine PrP gene expression, it is important to be able to isolate and quantify the level of endogenous PrP^c from cell culture extracts and tissue samples.

The isolation of PrP^{sc} from tissue samples is a well-established technique (Hope *et al.*, 1986), however, methods for the isolation of PrP^c protein are less well defined (Pan, Stahl & Prusiner, 1992). Methods used for the isolation of PrP^{sc} tend to use very stringent techniques that would easily degrade PrP^c. By definition PrP^c is far less stable than PrP^{sc} and is easily degraded, for example, PrP^c is sensitive to digestion by proteinase K (PK), whilst PrP^{sc} is partially resistant to PK digestion (Oesch *et al.*, 1985). In addition, the half-life of PrP^c has been shown to be much shorter than that of PrP^{sc} (Caughey, 1993). The level of PrP^c expression has been shown to vary in different cell types and tissues, with the highest amounts of PrP^c in

sheep in the brain (Caughey *et al.*, 1988, Oesch *et al.*, 1985). Within the brain the highest concentrations of PrP^c are found in neurones and astrocytes (Harris *et al.*, 1993, Raeber, Race, Brandner *et al.*, 1997). In contrast, the lowest levels of PrP^c are found in peripheral tissues, such as the liver (Caughey *et al.*, 1988, Horiuchi *et al.*, 1995, Moudjou, Frobert, Grassi *et al.*, 2001). Previous studies showed that the immortalised ovine cell cultures sA80BR and pA80BR used in the work in this thesis, contained both neurones and glial cells (Sections 2.5.1 & 2.5.3), whilst the Icelandic sheep cerebellum derived cell culture IS120Cer (Sections 2.5.2 & 2.5.3) contained only neurons (Marshall, 2000), although the relative proportion of these cell types may vary and could be influenced by culturing conditions. Whilst it has been shown that of brain derived cells, neurones and astrocytes express the highest levels of PrP^c, the amount of PrP^c expression in glial cells remains unclear (Harris *et al.*, 1993, Raeber *et al.*, 1997). It was therefore necessary to determine the levels of endogenous PrP^c expression in the ovine cell cultures prior to transient transfection studies with recombinant PrP^c.

For the study in this thesis it was decided to develop novel methods of cell lysis with the specific aim of extracting protein from ovine cell cultures. Marshall (2000) investigated a number of different methods of isolating endogenous PrP^c from cultured cells, including immunoprecipitation, detergent extraction, and chloroform/methanol extraction. Endogenous PrP^c could not be detected from cultured cell extracts (approximately 2×10^7 cells) without a prior concentrating step, and PrP^c was not detected in extracts prepared by detergent or chloroform/methanol extraction (Marshall, 2000). Immunoprecipitation was identified as being the most promising method of isolating endogenous PrP^c from the cultured cell extracts, although the overall level of PrP^c isolated was still low (Marshall, 2000). In order to detect endogenous PrP^c it was important to develop a system that would be capable of isolating sufficient quantities of PrP^c from the available cultured cells. It was expected that once established the same system could be used to isolate recombinant PrP^c from transiently transfected cell cultures, thus providing a method of assessing how well reporter gene constructs expressed recombinant PrP^c (Chapter 4). Immunoprecipitation not only allowed the efficient isolation of the target antigen, it also resulted in the disposal of non-target protein from the sample, thus reducing

contamination, and provided a sample concentration step. It was therefore, decided to use immunoprecipitation to isolate and quantify the level of endogenous PrP^c in the cultured cells in these studies.

For all of the samples used in this study cells cultured in single wells of a six well plate (approximately 2 x 10^5 cells per well) were used for the detection of endogenous PrP^c. The main benefits of this culture format are that samples are easier to handle, allowing faster treatment of cultures, thus providing a more uniform management of cells. In addition, it was expected that the possibility of variations in the number of cells used in each sample would be reduced with the six well plate format and this would prove to be very important when cultured cells were transiently transfected with ovine PrP mini-gene DNA (Chapter 4). In order to improve the overall PrP^c recovery from the cultured cells different methods of cell lysis specifically designed for mammalian cells (mammalian protein extraction reagent (M-per) and mammalian membrane protein extraction reagent (Mem-per), Pierce, UK) were utilised in these studies. Immunoprecipitation will only work if reliable antibodies specific for the target antigen are available. Two PrP specific polyclonal antibodies (1A8 & 1B3) (Farquhar *et al.*, 1989) were used in this study and in addition, a range of monoclonal antibodies were tested for their ability to capture PrP^c in immunoprecipitation from cell extracts. The immunoprecipitation technique was further enhanced by utilising a range of different sepharose matrices and buffering systems.

3.2 Results

3.2.1 Development of methods for lysis of murine N2a cells

Novel methods for isolating PrP^c were developed in the murine N2a cells. The main reason for using the N2a cells was that methods for lysing them had already been successfully developed, and could be used as a basis for the further development of PrP^c extraction techniques. Comparisons were made between the lysis reagent used in previous studies, i.e. reporter cell lysis buffer (RCLB, Roche, UK) (Marshall, 2000) and the mammalian protein extraction reagent system (M-per, Pierce, UK), in order to determine the effectiveness of each of the lysis reagents at

extracting PrP^c from N2a cells. Cell extracts were prepared from N2a cells cultured to confluency in a single well of a six well plate (approximately 2 x 10^5 cells) using either RCLB or M-per (Section 2.5.9). The concentration of total protein within each lysate was determined by comparing the absorbance values at 595 nm obtained for each sample against a protein concentration standard curve (Section 2.5.10). The experiment was performed in total three times and the results were combined to provide a mean value for each protein extraction method (Figure 3.1). Cell extracts prepared with M-per contained on average 0.14 mg/ml total protein whilst those prepared with RCLB contained on average 0.019 mg/ml protein (Figure 3.1). A slight increase to 0.15 mg/ml was observed with M-per in the presence of proteinase inhibitors, whilst the level of total protein almost doubled to 0.035 mg/ml in cell extracts prepared with RCLB in the presence of the same inhibitor solution (Figure 3.1). Therefore a four-fold increase in total protein concentration was observed with M-per, as compared to RCLB in the presence of proteinase inhibitors (Figure 3.1).

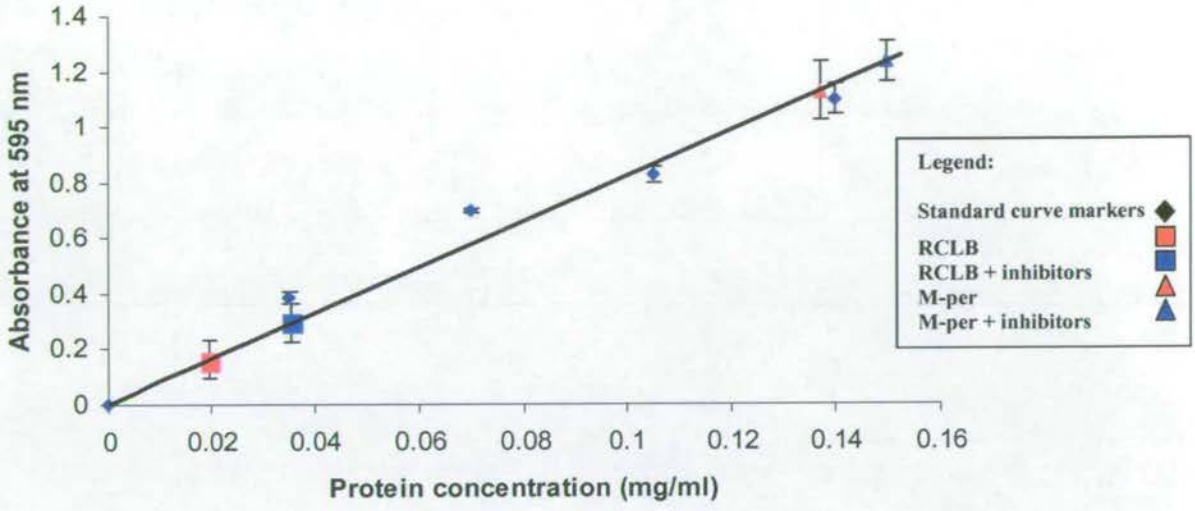


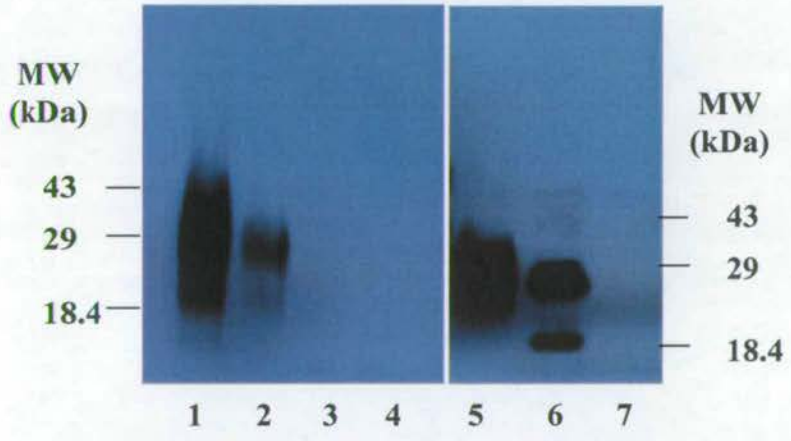
Figure 3.1 *Comparison of M-per and RCLB protein extraction systems*

Two different cell lysis reagents mammalian protein extraction reagent (M-per) and reporter cell lysis buffer (RCLB) were used to isolate total protein from murine neuroblastoma (N2a) cells. Protein extractions were performed both with and without the addition of proteinase inhibitors.

3.2.2 Immunoprecipitation of purified PrP^{sc} samples and endogenous PrP^{c} from murine N2a cell extracts and their analysis by Chemiluminescence blotting

To develop the immunoprecipitation technique ovine brain tissue samples positive for PrP^{sc} were used. The main benefits of using PrP^{sc} samples are that they are of a consistent concentration, are more stable than PrP^{c} and give a strong reaction with the monoclonal antibody 6H4 in a Western blot. The polyclonal antibodies 1A8 and 1B3 (Farquhar *et al.*, 1989) were used to precipitate PrP (Section 2.7.4). Throughout this chapter precipitated samples were separated by SDS-PAGE and PrP was detected immunologically by Western blotting (Section 2.8) using the primary antibody 6H4 at a 1:10,000 dilution (Section 2.8.2) and a peroxidase conjugated affinipure rabbit anti-mouse IgG at a 1:25,000 dilution as the secondary antibody (Section 2.8.3). Proteinase K (PK) treated purified PrP^{sc} ovine brain samples were prepared by Kasia Sobotnicki, NPU using the method described by Hope, Multhaup,

Reekes *et al.* (1988) and used as positive control samples. The purified PrP^{sc} brain samples (100 mg starting wet brain tissue weight) were re-suspended in 100 µl SDS loading buffer and 20 µl (equivalent to 20 mg tissue) of this was loaded as a positive control. The positive controls were further diluted 1 in 3, and 1 in 9 with loading buffer, and again 20 µl (equivalent to 7 mg, or 2 mg of tissue, respectively) of the diluted sample was loaded. Non-immunoprecipitated purified PrP^{sc} samples were analysed by SDS-PAGE and Western blotting with 6H4 (Section 2.8), alongside purified PrP^{sc} samples that had been diluted in TBST (400 µl) and immunoprecipitated (Section 2.7.3) as normal with the polyclonal antibody 1A8 (Lanes 3-4, Figure 3.2).



KEY: Lane 1, Purified PrP^{sc} control sample treated with proteinase K (PK) (equivalent to 20 mg brain tissue); Lane 2, 1 in 3 dilution of Lane 1 sample (equivalent to 7 mg brain tissue); Lanes 3-4, Purified PrP^{sc} control samples, pre-adsorbed & immunoprecipitated with 1A8 (equivalent to 20 mg brain tissue); Lane 5, Purified PrP^{sc} control sample (equivalent to 2 mg brain tissue); Lane 6, Purified PrP^{sc} control sample (equivalent to 20 mg brain tissue) diluted in 400 µl TBST, immunoprecipitated with 1A8 without pre-adsorption step; Lane 7, Purified PrP^{sc} control sample (equivalent to 20 mg brain tissue) diluted in 400 µl TBST, pre-adsorbed with protein A sepharose & then immunoprecipitated with 1A8. PrP^{sc} positive controls used in lanes 5 & 6 were from separate samples.

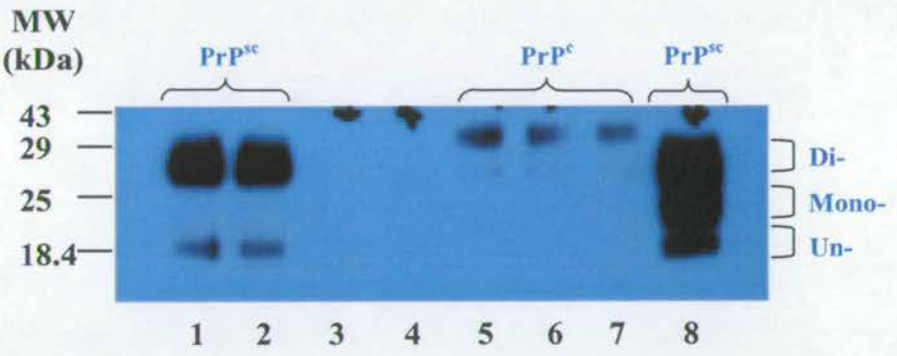
Figure 3.2 *PrP^{sc} positive controls: Immunoprecipitated & non-immunoprecipitated with 1A8 positive control samples*

Western blot shows PrP^{sc} positive ovine brain samples either pre-adsorbed or non pre-adsorbed with protein A sepharose and then immunoprecipitated with anti-PrP polyclonal antibody 1A8 and detected with anti-PrP monoclonal antibody 6H4 or loaded directly without an immunoprecipitation step.

PrP^{sc} immunoprecipitated from purified PrP^{sc} brain samples was not detected, however, PrP^{sc} was clearly visible in non-immunoprecipitated samples (Lanes 1-2, Figure 3.2). The possibility that this was due to the pre-adsorption of the extracts with protein A sepharose was investigated. Purified PrP^{sc} brain samples were immunoprecipitated both with and without the initial pre-adsorption step (Section 2.7.3), the results were separated by SDS-PAGE and analysed by Western blotting with 6H4 (Section 2.8) (Lanes 5-7, Figure 3.2). The pre-adsorption of the purified PrP^{sc} samples with protein A sepharose resulted in the removal of PrP^{sc}, probably via non-specific binding to the sepharose matrix (Lane 7, Figure 3.2). As a consequence of this observation, endogenous PrP^{c} isolated from N2a cell extracts prepared with M-per and RCLB was compared with methods omitting this initial pre-adsorption step. Finally, the PrP^{sc} sample (equivalent to 20 mg brain tissue) diluted in 400 µl of TBST appeared to be successfully immunoprecipitated with 1A8, indicating that the immunoprecipitation step had worked and had resulted in the concentration of PrP^{sc} (Lane 6, Figure 3.2).

Attempts were made to isolate endogenous PrP^{c} from the murine N2a cell extracts using purified PrP^{sc} brain samples as a positive control. During the extraction of PrP^{sc}, the samples are treated with PK, which results in the isolation of a truncated form of PrP^{sc}. Treatment with PK results in the degradation of endogenous PrP^{c}, which is sensitive to the action of proteinase K and when visualised on a gel, PrP^{c} is located at a higher molecular weight band than the truncated PrP^{sc} (compare lanes 7 (PrP^{c}) & 8 (PrP^{sc}), Figure 3.3). In addition, PrP is normally seen on Western blots as three separate glycoforms, un-, mono- and di-glycosylated PrP. Furthermore the mono-glycosylated band is actually a doublet as there are two mono-glycosylated forms of PrP. For each sample a single well of a six well plate of confluent N2a cells (approximately 2×10^5 cells) was lysed with RCLB (Section 2.5.9.2) or M-per (Section 2.5.9.3). Endogenous PrP^{c} was immunoprecipitated (Section 2.7.3) with the polyclonal antibody 1A8 and samples were separated by SDS-PAGE and analysed by Western blotting with 6H4 (Section 2.8). Comparisons of the RCLB and M-per extraction systems showed that PrP^{c} was successfully immunoprecipitated with 1A8 from N2a cell extracts prepared with M-per (Lanes 5-7, Figure 3.3), however, no PrP^{c} was detected in the cell extracts

prepared with RCLB (Lanes 3 & 4, Figure 3.3). PrP^{sc} was successfully detected from the diluted purified PrP^{sc} samples (Lanes 1 & 2, Figure 3.3). It was noted that only two of the three glycoforms of PrP^{c} and PrP^{sc} were visible in the immunoprecipitated samples (Lanes 1, 2, 5, 6 & 7, Figure 3.3). It appears that the mono-glycosylated forms are missing in the immunoprecipitated PrP^{sc} samples (Lanes 1 & 2, Figure 3.3), whilst the unglycosylated band appears to be absent in the immunoprecipitated PrP^{c} samples (Lanes 5-7, Figure 3.3).



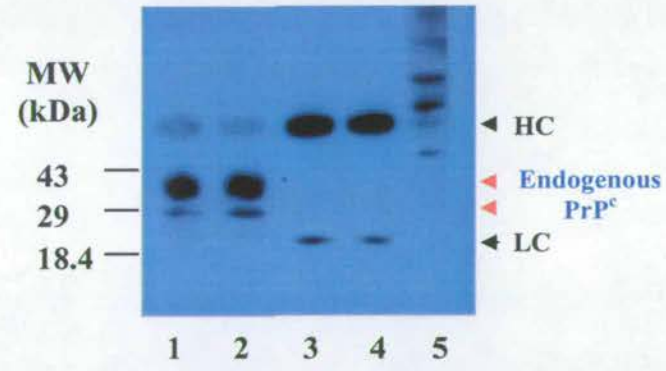
KEY: Lanes 1-2, Purified PrP^{sc} control samples treated with PK (equivalent to 20 mg brain tissue) diluted in 400 µl TBST & immunoprecipitated with 1A8; Lanes 3-4, N2a cell extracts prepared with RCLB, immunoprecipitated with 1A8; Lanes 5-7, N2a cell extracts prepared with M-per, immunoprecipitated with 1A8; Lane 8, Purified PrP^{sc} control sample (2 mg). PrP is observed as three different glycoforms by Western blot analysis: Di- = di-glycosylated PrP, Mono- = mono-glycosylated PrP, Un- = un-glycosylated PrP.

Figure 3.3 *N2a cell extracts: PrP^{c} immunoprecipitated with 1A8 from extracts prepared with either M-per or RCLB*

Western blot shows endogenous PrP^{c} isolated from N2a cell extracts produced with either M-per or RCLB lysis agents and immunoprecipitated with anti-PrP polyclonal antibody 1A8. Samples were loaded alongside PrP^{sc} positive ovine brain samples either immunoprecipitated with 1A8 or loaded directly without an immunoprecipitation step. Western blot was detected with the anti-PrP monoclonal antibody 6H4. Endogenous PrP^{c} can be clearly seen in the M-per samples but not at all in the RCLB samples.

The use of two other anti-PrP antibodies, 1B3 (polyclonal) and 6H4 (monoclonal) as precipitating antibodies was investigated (Figure 3.4). As can be seen from Figure 3.4, the amount of PrP^{c} detected in N2a cell extracts

immunoprecipitated with 1B3 was comparable to that observed with 1A8 (Lanes 1 & 2, Figure 3.4). Again only the di- and mono-glycosylated PrP^c bands were present in extract immunoprecipitated with 1B3 (Lane 2, Figure 3.4). No endogenous PrP^c was detected in the raw N2a cell extract (30 µl of total 400 µl N2a extract loaded), indicating that the concentrating step provided by the immunoprecipitation reaction was required in order to be able to detect endogenous PrP^c from the N2a cell extracts (Lane 5, Figure 3.4). The use of 6H4 failed to isolate any detectable PrP^c from the N2a cell extracts, however, two additional bands were observed (25 and 50 kDa), these are presumably the light (LC) and heavy chains (HC) of the denatured precipitating 6H4 antibody (Lanes 3 & 4, Figure 3.4). These bands are visible due to cross reactivity of the precipitating antibody with the secondary antibody (rabbit anti-mouse IgG). It will also react lightly with the rabbit derived polyclonal antibodies 1A8 and 1B3, and this can be seen as much fainter bands at 25 and 50 kDa (Lanes 1 & 2, Figure 3.4). The polyclonal antibody 1B3 is in very short supply, and so most of the immunoprecipitations in this study were carried out with 1A8.



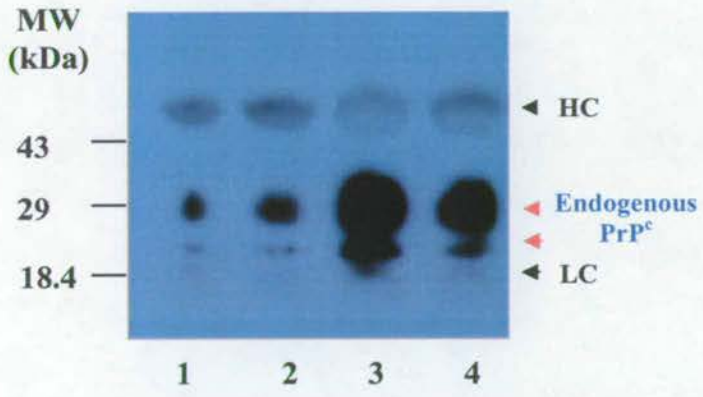
KEY: N2a cell extracts prepared with M-per and immunoprecipitated with the stated antibody. Lane 1, 1A8; Lane 2, 1B3; Lanes 3-4, 6H4; Lane 5, 30 µl aliquot of a raw non-immunoprecipitated N2a cell extract (400 µl total) produced with M-per. PrP^c marked with red arrowheads. LC = light chain of precipitating antibody, HC = heavy chain.

Figure 3.4 *N2a cell extracts: PrP^c immunoprecipitated from M-per extracts with 1A8, 1B3 or 6H4*

Western blot shows endogenous PrP^c isolated from N2a cell extracts prepared with M-per and immunoprecipitated with the anti-PrP polyclonal antibodies 1A8 or 1B3, or with the anti-PrP monoclonal antibody 6H4. Samples were then detected with 6H4. As can be seen endogenous PrP^c was successfully isolated using the 1A8 and 1B3 polyclonal antibodies but not with the 6H4 monoclonal antibody (Red arrowheads).

3.2.3 Analysis of different sepharose matrices for immunocomplex capture

The immunoprecipitations to this stage had been carried out using a protein A sepharose matrix. The effectiveness of a protein G sepharose matrix for the PrP^c immunoprecipitations was tested, as it has been shown to react more strongly with antibodies of the same IgG sub-type as 1A8 & 1B3, and with the same antibody class and sub-types as many monoclonal antibodies. The use of a protein G sepharose matrix resulted in a significant increase in the level of endogenous PrP^c isolated from the N2a cell extracts using 1A8 and following this result a protein G sepharose matrix was used for most of the immunoprecipitations in this study (Figure 3.5).



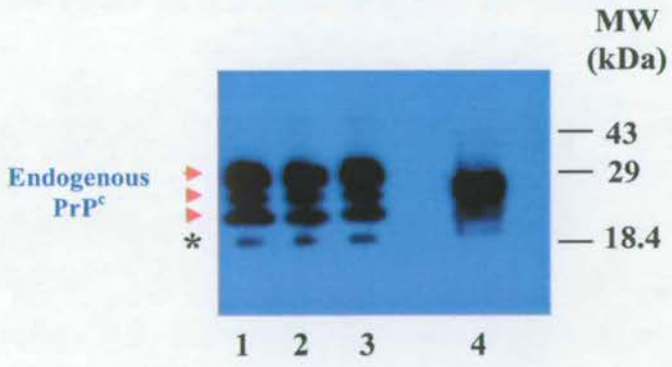
KEY: Lanes 1-2, N2a cell extracts prepared with M-per, immunoprecipitated with 1A8 using a protein A sepharose matrix; Lanes 3-4, N2a cell extracts prepared with M-per, immunoprecipitated with 1A8 using a protein G sepharose matrix. PrP^c marked with red arrowheads. LC = light chain of precipitating antibody, HC = heavy chain.

Figure 3.5 *N2a cell extracts: PrP^c immunoprecipitated from M-per extracts with 1A8, using two different sepharose matrices*

Western blot shows endogenous PrP^c (Red arrowheads) isolated from M-per produced N2a cell extracts and immunoprecipitated using the anti-PrP polyclonal antibody 1A8 with either a protein A or protein G sepharose matrix and detected with the anti-PrP monoclonal antibody 6H4. As can be seen the use of the protein G sepharose resulted in the isolation of more PrP^c than the protein A sepharose matrix.

3.2.4 Comparison of M-per and Mem-per cell lysis systems

PrP^c is a membrane-associated protein and as such it was decided to test a cell extraction technique that was specific for membrane proteins. The extraction system used until now was M-per, which is a mammalian-cell specific lysis agent. However, as M-per isolates both cytoplasmic and membrane attached proteins, it was decided to try the Mem-per system. N2a cell extracts were prepared with Mem-per (Section 2.5.9.4), immunoprecipitated with 1A8 using a protein G sepharose matrix (Section 2.7.3), samples were separated by SDS-PAGE and analysed by Western blotting (Section 2.8) using 6H4 (Figure 3.6).



KEY: Lanes 1-3, N2a cell extracts prepared with Mem-per, and immunoprecipitated with 1A8 using a protein G sepharose matrix; Lane 4, Purified PrP^{sc} control sample treated with PK (equivalent to 2 mg brain tissue). PrP^c marked with red arrowheads. * marks the location of an additional band which may represent a processed or degraded PrP^c product.

Figure 3.6 *N2a cell extracts: PrP^c immunoprecipitated from Mem-per extracts with 1A8*

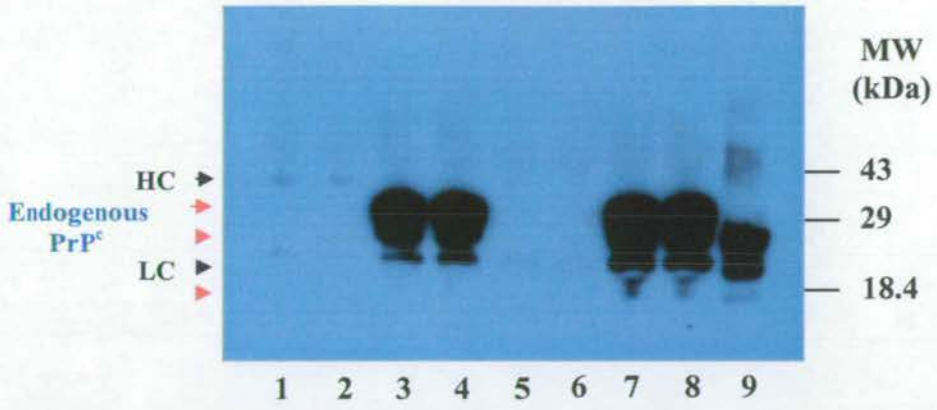
Western blot shows endogenous PrP^c (Red arrowheads) isolated from Mem-per produced N2a cell extracts immunoprecipitated with the anti-PrP polyclonal antibody 1A8 using a protein G sepharose matrix and detected with the anti-PrP monoclonal antibody 6H4.

As can be seen in Figure 3.6, all three PrP^c glycoforms were immunoprecipitated with 1A8 and successfully detected using 6H4 from the Mem-per produced N2a cell extracts. A further band was observed (marked with *, Lanes

1-3, Figure 3.6) although it is not clear if this band represents cross reactivity with the precipitating antibody light chain or a processed or degraded PrP^c product.

3.2.5 Immunoprecipitation of endogenous PrP^c from murine brain tissue samples

The immunoprecipitation system was developed further by testing its ability to successfully isolate PrP^c from uninfected murine brain tissue samples. PrP null transgenic mice, which do not express endogenous PrP^c, and PrP wild type mice are available at the NPU, and brain samples were obtained from each of these animals and processed identically. Tissue extracts were produced as described in section 2.5.9.1, and protein extracts were prepared with either M-per (Section 2.5.9.3) or Mem-per (Section 2.5.9.4), in the presence of proteinase inhibitors and immunoprecipitated with 1A8 using a protein G sepharose matrix (Section 2.7.3). Samples were separated by SDS-PAGE and analysed by Western blotting (Section 2.8) using 6H4 (Figure 3.7).



KEY: Lanes 1-2, PrP null mouse brain tissue extracts prepared with M-per, and immunoprecipitated with 1A8; Lanes 3-4, PrP wild-type mouse brain tissue extracts prepared with M-per, and immunoprecipitated with 1A8; Lanes 5-6, PrP null mouse brain tissue extracts prepared with Mem-per, and immunoprecipitated with 1A8; Lanes 7-8, PrP wild-type mouse brain tissue extracts prepared with Mem-per, and immunoprecipitated with 1A8; Lane 9, Purified PrP^{sc} control sample treated with (PK) (equivalent to 2 mg brain tissue). PrP^{c} marked with red arrowheads. LC = light chain of precipitating antibody, HC = heavy chain.

Figure 3.7 *Murine brain extracts: PrP^{c} immunoprecipitated from tissue extracts prepared with either M-per or Mem-per*

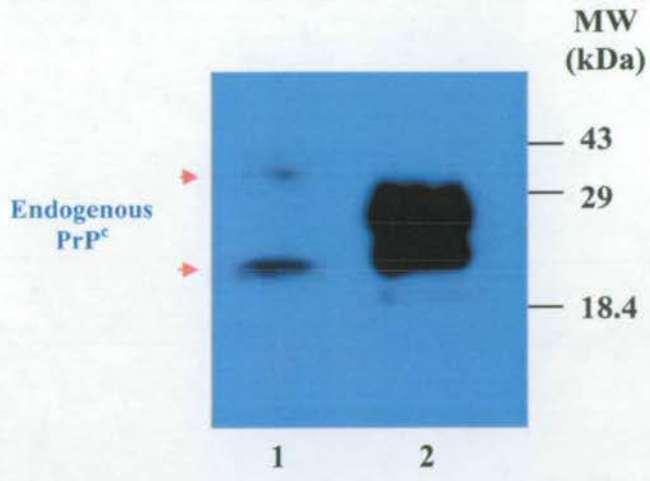
Western blot shows endogenous PrP^{c} (Red arrowheads) isolated from M-per and Mem-per produced PrP null and wild-type mouse brain extracts immunoprecipitated with the anti-PrP polyclonal antibody 1A8 using a protein G sepharose matrix and detected using the anti-PrP monoclonal antibody 6H4. As can be seen PrP^{c} was only observed in the wild-type mouse brain extracts.

As can be seen from Figure 3.7 endogenous PrP^{c} (red arrowheads, Figure 3.7) was successfully immunoprecipitated from the wild-type mouse brain tissue (Lanes 3, 4, 7 & 8), whilst as expected the PrP null mouse brain tissue showed no detectable PrP^{c} (Lanes 1, 2, 5 & 6). No significant difference was observed in the level of PrP^{c} recovery between M-per and Mem-per produced tissue extracts (Figure 3.7). However, all three glycoforms may have been isolated in the Mem-per produced wild-type mouse brain tissue extract (Lanes 7 & 8, Figure 3.7), whilst the un-glycosylated band is less clear in the M-per produced wild-type brain extract (Lanes 3 & 4, Figure 3.7). This result is consistent with that seen with PrP^{c} extracted with Mem-per from the N2a cell extracts where all three glycoforms were clearly detected

(Figure 3.6). In addition, no PrP^c was detected in Mem-per produced wild type mice brain extracts, which were extracted without a precipitating antibody (data not shown). This result demonstrated that the immunoprecipitation system was working effectively as a sample concentration step, and that the presence of a precipitating antibody was crucial. As would be expected endogenous PrP^c was undetectable in murine liver tissue (data not shown).

3.2.6 Extraction and analysis of endogenous PrP^c from ovine cell culture extracts and the determination of background levels of PrP^c expression

The extraction and immunoprecipitation techniques developed with the use of purified PrP^{sc} control samples, murine N2a cell extracts, and murine brain tissue samples were used to isolate endogenous PrP^c from ovine cell culture extracts. Cell extracts were prepared from a single, confluent well of a six well plate of sA80BR cells (approximately 2 x 10^5 cells) using Mem-per (Section 2.5.9.4), and immunoprecipitations were carried out with 1A8 (Section 2.7.3) using a protein G sepharose matrix. Samples were separated by SDS-PAGE and analysed by Western blotting (Section 2.8) using 6H4 (Figure 3.8). The results showed that PrP^c was clearly detectable (albeit at low levels) in sA80BR cell extracts prepared with the Mem-per system (Lane 1, Figure 3.8). The fact that this sample was first immunoprecipitated with a PrP specific antibody (1A8), and then separately detected with another PrP specific antibody (6H4), indicates that these bands are very likely to be PrP^c. However, only two glycoforms were detected in the ovine sA80BR cell extract prepared with Mem-per (Lane 1, Figure 3.8). The molecular weights of these bands are approximately 33 kDa and 22 kDa, respectively (Lane 1, Figure 3.8), indicating that they may be the di- and un-glycosylated bands of PrP^c, respectively. Alternatively the lower band could be a processed or degraded PrP^c product.

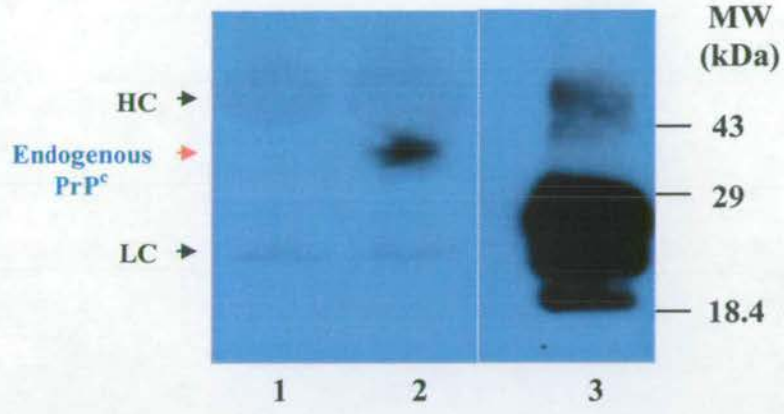


KEY: Lane 1, sA80BR cell extract prepared with Mem-per, and immunoprecipitated with 1A8; Lane 2, Purified PrP^{sc} control sample treated with PK (equivalent to 2 mg brain tissue). Red arrowheads indicate PrP^{c} bands.

Figure 3.8 *Ovine sA80BR extracts: PrP^{c} immunoprecipitated with 1A8 from Mem-per extracts*

Western blot shows endogenous PrP^{c} (Red arrowheads) isolated from Mem-per produced ovine sA80BR (isolated from a sheep of a scrapie susceptible genotype) neuronal cell extract, immunoprecipitated with the anti-PrP polyclonal antibody 1A8 using a protein G sepharose matrix and detected with the anti-PrP monoclonal antibody 6H4.

Further immunoprecipitations were performed using Mem-per (Section 2.5.9.4) cell extracts produced from the Icelandic sheep cell cultures IS120Cer and IS120Liv (approximately 2 x 10^5 cells). Extracts were immunoprecipitated with 1A8 (Section 2.7.3), using a protein G sepharose matrix, samples were separated by SDS-PAGE, and analysed by Western blotting (Section 2.8) using 6H4 (Figure 3.9). The results showed that endogenous PrP^{c} (Lane 2, Figure 3.9) was successfully isolated from the IS120Cer cell extracts, although only one glycoform was observed (red arrowhead, Figure 3.9). This is likely to be the di-glycosylated form of PrP^{c} as its has a molecular weight of approximately 33 kDa. Again, the fact that these samples were immunoprecipitated and detected with separate PrP specific antibodies indicates that the observed band is very likely to be that of PrP^{c}. In contrast no endogenous PrP^{c} was detected in the extracts of the liver-derived cell culture IS120Liv (Figure 3.9).



KEY: Lane 1, IS120Liv cell extract prepared with Mem-per, and immunoprecipitated with 1A8; Lane 2, IS120Cer cell extract prepared with Mem-per, and immunoprecipitated with 1A8; Lane 3, Purified PrP^{sc} control sample treated with PK (equivalent to 2 mg brain tissue). PrP^{c} marked with red arrowheads. LC = light chain of precipitating antibody, HC = heavy chain.

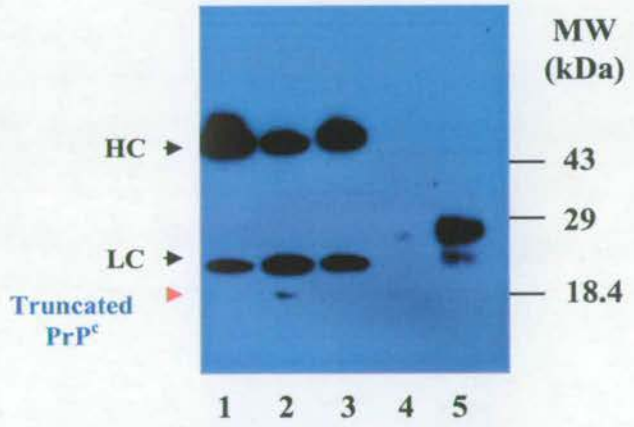
Figure 3.9 *Ovine IS120 cell extracts: PrP^{c} immunoprecipitated with 1A8 from Mem-per extracts*

Western blot shows endogenous PrP^{c} (Red arrowhead) isolated from Mem-per produced ovine IS120Liv (liver) and IS120Cer (cerebellum) cell extracts, immunoprecipitated with the anti-PrP polyclonal antibody 1A8 using a protein G sepharose matrix and detected with the anti-PrP monoclonal antibody 6H4. As can be seen PrP^{c} was detected in the IS120Cer cell extract but not in the IS120Liv cell extract.

3.2.7 Characterisation of PrP-specific monoclonal antibodies as immunoprecipitating antibodies

A number of mouse derived PrP-specific monoclonal antibodies showing cross reactivity with PrP from a number of different mammalian species (Table 2.6) are available in the Institute for Animal Health (IAH). It was expected that the use of these antibodies would improve the immunoprecipitation system further, and their availability raised the possibility of using an immunoprecipitating cocktail of monoclonal antibodies to give multiple epitope recognition of PrP. Two sets of antibodies are available; set one consists of BE12, AH6 and AG4 (Jacquie Manser, IAH, Compton) and these antibodies recognise N-terminal epitopes on PrP (Table

2.6). Set two consists of eight antibodies named AB1-AB8 (Dr Sandra McCutcheon, IAH, Compton) and these antibodies recognise C-terminal epitopes on PrP (Table 2.6). The ability of these antibodies to precipitate endogenous PrP^c from the murine N2a cells was assessed in the immunoprecipitation system developed during this study (Section 2.7.3). N2a cell extracts (approximately 2 x 10^5 cells) prepared with Mem-per (Section 2.5.9.4) were immunoprecipitated with the appropriate monoclonal antibody. Samples were separated by SDS-PAGE, and analysed by Western blotting with 6H4 (Section 2.8). The results of these immunoprecipitations are shown in Figures 3.10 & 3.11.

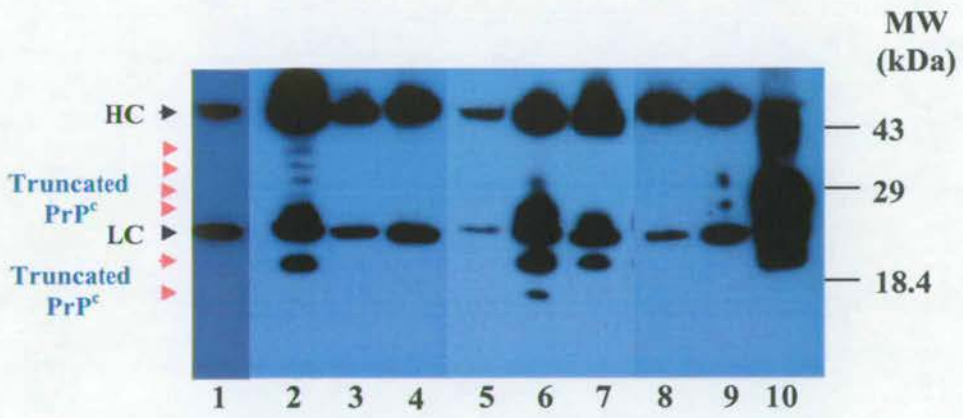


KEY: N2a cell extracts prepared with Mem-per, and immunoprecipitated with the stated monoclonal antibody. Lane 1, BE12; Lane 2, AH6; Lane 3, AG4; Lane 4, 1A8; Lane 5, Purified PrP^{sc} control sample treated with PK (equivalent to 2 mg brain tissue). PrP^c marked with red arrowheads. LC = light chain of precipitating antibody, HC = heavy chain.

Figure 3.10 *N2a cell extracts: PrP^c immunoprecipitated from Mem-per extracts, with PrP-specific monoclonal antibodies (set one)*

Western blot shows endogenous PrP^c (Red arrowheads) isolated from Mem-per produced N2a cell extracts immunoprecipitated with the anti-PrP monoclonal antibodies BE12, AH6 or AG4 (directed towards epitopes at the PrP N-terminal region) using a protein G sepharose matrix and detected with the anti-PrP monoclonal antibody 6H4. The PrP^c isolated appeared to be of a truncated form of approximately 20 KDa.

As can be seen from Figure 3.10, immunoprecipitations with the set one antibodies showed mixed results. The monoclonal antibodies BE12 and AG4 failed to immunoprecipitate any detectable PrP^c from the N2a cell extracts (Lanes 1 & 3, Figure 3.10). The monoclonal antibody AH6 showed promise with a small amount of PrP^c detectable (Lane 2, Figure 3.10), however, the PrP^c observed appeared to be slightly truncated with an approximate molecular weight of 20 kDa (red arrowhead, Figure 3.10). In addition, strong bands were observed at 25 and 50 kDa, which presumably represent cross reactivity of the light (LC) and heavy chains (HC) of the precipitating antibody with the secondary antibody.



KEY: N2a cell extracts prepared with Mem-per, and immunoprecipitated with the stated monoclonal antibody. Lane 1, AB1; Lane 2, AB2; Lane 3, AB3; Lane 4, AB4; Lane 5, AB5; Lane 6, Cocktail of all set two monoclonal antibodies; Lane 7, AB6; Lane 8, AB7; Lane 9, AB8; Lane 10, Purified PrP^{sc} control sample treated with PK (equivalent to 2 mg brain tissue). PrP^c marked with red arrowheads. LC = light chain of precipitating antibody, HC = heavy chain. Red arrows indicate location of possible truncated PrP^c bands.

Figure 3.11 *N2a cell extracts: PrP^c immunoprecipitated from Mem-per extracts with PrP-specific monoclonal antibodies (set two)*

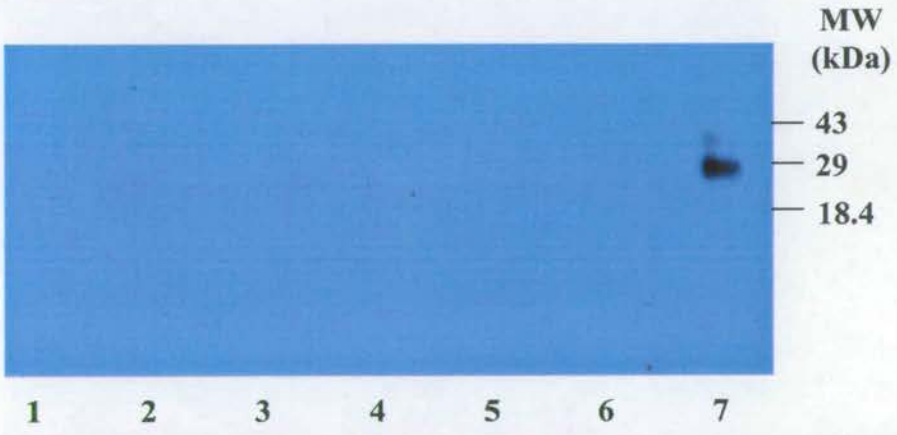
Western blot shows endogenous PrP^c (Red arrowheads) isolated from Mem-per produced N2a cell extracts using the anti-PrP monoclonal antibodies AB1-AB8 (directed towards epitopes at the PrP C-terminal region) using a protein G sepharose matrix and detected using the anti-PrP monoclonal antibody 6H4. The PrP^c isolated appeared to be of a truncated form of approximately 20 KDa.

The set two antibodies showed mixed results (Figure 3.11). Positive results were observed with AB2, AB6 and AB8, whilst AB1, AB3, AB4, AB5 and AB7 failed to show any detectable PrP^c (Figure 3.11). The set two antibodies that isolated endogenous PrP^c from the N2a cell extracts showed very promising results with strong levels of PrP^c clearly detectable (AB2, AB6 & AB8) (Lanes 2, 7 & 9, Figure 3.11). In addition, a cocktail of all of the set two monoclonal antibodies added together in a single immunoprecipitation reaction resulted in a strong signal for PrP^c (Lane 6, Figure 3.11). This reaction was very likely to be the result of the antibodies AB2, AB6 and AB8 as these showed individual reactivity with PrP^c in this system. Again the endogenous PrP^c isolated from the N2a cells during these immunoprecipitations appeared to be of a truncated form, of approximately 20 kDa (red arrowheads, Figure 3.11). Cross reactivity between the secondary antibody and the light (LC) and heavy chains (HC) of the precipitating antibody produced strong bands at 25 and 50 kDa (Figure 3.11).

3.2.8 Coupling of the AB2, AB6 & AB8 monoclonal antibodies to an amino-link matrix

As seen with all of the mouse derived monoclonal antibodies, strong bands due to the light and heavy chains of the precipitating antibodies (25 and 50 kDa, respectively) were observed (LC & HC, Figures 3.10 & 3.11). The PrP^c detected here appeared to be of a truncated form (approximately 20 kDa) and unfortunately this meant that the PrP^c bands fell across the line of the precipitating antibody light chain. This made it very difficult to clearly identify the PrP^c bands and it would have been desirable to remove these heavy and light chain bands from the samples prior to loading. These bands appeared because after the immunoprecipitation reaction the immunocomplexes were denatured in order to release the antigen, however this caused the dissociation of the precipitating antibody chains. The protein G sepharose matrix was pelleted by centrifugation, leaving the isolated PrP^c and the heavy and light chains of the precipitating antibody free in the supernatant. In order to prevent this it was necessary to use a matrix that would not be denatured by heating, but that would still allow the immunoprecipitated PrP^c to be released. It was decided to use an amino-link matrix (Pierce, UK) which would enable the permanent coupling of

the monoclonal antibodies. The bonds formed between the amino-link matrix and the precipitating antibody are not broken by heat denaturation, however the antigen (PrP^c) can be released from the antibody/matrix by using a specific elution buffer (Pierce, UK). It was hoped that the use of this matrix would allow PrP^c to be immunoprecipitated with the monoclonal antibodies, whilst preventing the release of the antibody heavy and light chains into the SDS-PAGE samples. The set two antibodies that proved successful in precipitating PrP^c from the N2a cells (AB2, AB6 & AB8) were individually coupled to the amino-link matrix, following the manufacturers instructions. Immunoprecipitations (Section 2.7.3) were performed using these antibody/matrix combinations, and samples were separated by SDS-PAGE and analysed by Western blotting (Section 2.8) using 6H4 (Figure 3.12).



KEY: N2a cell extracts prepared with Mem-per, and immunoprecipitated with the stated monoclonal antibody coupled to an amino-link matrix. Lanes 1-2, AB2; Lanes 3-4, AB6; Lanes 5-6, AB8; Lane 7, Purified PrP^{sc} control sample treated with PK (equivalent to 2 mg brain tissue).

Figure 3.12 *N2a cell extracts: PrP^c immunoprecipitated from Mem-per extracts with PrP-specific monoclonal antibodies directly coupled to an amino-link matrix*

Western blot shows an attempt to isolate endogenous PrP^c from Mem-per produced N2a cell extracts immunoprecipitated with the anti-PrP monoclonal antibodies AB2, AB6 or AB8 covalently attached to an amino-link matrix in order to be able to isolate PrP without the contaminating bands of the precipitating antibody light and heavy chains. Samples were detected using the anti-PrP monoclonal antibody 6H4. As can be seen no endogenous PrP^c was detected, neither were the light or heavy chains of the precipitating antibodies.

As can be seen from Figure 3.12 the immunoprecipitations carried out with the amino-linked monoclonal antibodies failed to isolate any detectable PrP^c from the N2a cell extracts. However, it was noted that no precipitating antibody bands (25 and 50 kDa, respectively) were observed on the Western blot, indicating that the antibodies may have been successfully coupled to the matrix.

3.3 Discussion

3.3.1 Technical aspects

Previous studies have shown that methods used successfully for the extraction of PrP^c from certain cell types do not necessarily work as effectively with other cell types (Madec, Groschup, Buschmann *et al.*, 1998, Marshall, 2000). Marshall (2000) was able to detect endogenous PrP^c in murine N2a cell extracts using immunoprecipitation, however the same method failed to extract detectable quantities of endogenous PrP^c from the ovine sA80BR cell extracts. It was hypothesised that differences in the concentration of endogenous PrP^c, the type of membrane association or cell and species-specific differences in membrane composition could explain the varying results observed in these studies (Madec *et al.*, 1998, Marshall, 2000). Previous studies had used approximately 2×10^7 cells for the detection of endogenous PrP^c, however, in this study cells cultured in a single well of a six well plate (approximately 2×10^5 cells) were used. The M-per system was chosen as a suitable cell lysis agent as it is designed specifically for this task and the results showed that M-per was a more effective lysis reagent than RCLB in terms of total protein recovery (Figure 3.1). The experiments also showed that M-per is far less reliant on the action of proteinase inhibitors than RCLB and that the use of M-per overall gave a four-fold increase in total protein recovery from the N2a cells. Applied to PrP detection, comparisons between M-per and RCLB showed that endogenous PrP^c protein was easily detectable in the M-per prepared N2a cell extracts whilst no PrP^c was detected in the RCLB prepared cell extracts (Figure 3.3). M-per had already been shown to recover far higher concentrations of total protein than RCLB, and this result showed that M-per was able to recover a detectable amount of PrP^c whilst RCLB was not. The amount of endogenous PrP^c visualised on

the Western blots in the present study was comparable to that shown by Marshall (2000). However, in the present study approximately 2 x 10^5 cells were used, whilst Marshall (2000) used approximately 2 x 10^7 cells. Therefore, the immunoprecipitation system developed in the present study appears to be two orders of magnitude more efficient at isolating PrP^c from the N2a cells than the isolation system used by Marshall (2000). Information regarding the sensitivity of the immunoprecipitation system developed during this study was provided by the use of purified PrP^{sc} samples (20 mg) diluted in 400 µl of TBST (0.05 mg/µl) and immunoprecipitated with 1A8. PrP^{sc} was clearly detectable from these diluted purified PrP^{sc} samples and the amount of PrP^{sc} detected, as judged by Western blot, was comparable to that observed with 2 mg of a purified PrP^{sc} sample loaded directly onto the same gel and detected in the same blot. Therefore, the immunoprecipitation step had concentrated the PrP^{sc} from the 400 µl of TBST, in which it was diluted (Lane 6, Figure 3.2; Lanes 1 & 2, Figure 3.3), although most of the PrP^{sc} was lost presumably in the immunoprecipitation wash steps. This experiment indicated that the immunoprecipitation system may have reached a detection ceiling, above which the amount of PrP^{sc} added would potentially have little effect on the outcome and this was possibly due to the availability of free 1A8 antibody. Therefore, the sensitivity of the immunoprecipitation system may be raised further by increasing the amount of free 1A8 antibody available.

For immunoprecipitations it is more desirable to use a polyclonal antibody, which is able to recognise multiple epitopes on the same protein molecule, thus increasing the chances of capturing the protein. The PrP specific monoclonal antibody 6H4 as an immunoprecipitating antibody failed to isolate PrP^c from the N2a cell lysates (Figure 3.4). 6H4 is a monoclonal antibody, as such it recognises only one epitope meaning that it is not an ideal antibody for immunoprecipitation. In contrast, both anti-PrP polyclonal antibodies 1A8 and 1B3 functioned equally as effectively as precipitating antibodies in this system (Figure 3.4).

A number of mouse-derived PrP specific monoclonal antibodies, produced at IAH, Compton, were analysed for their ability to isolate endogenous PrP^c from N2a cell extracts. In particular three of the antibodies (AB2, AB6 & AB8) successfully isolated endogenous PrP^c from the N2a cell extracts (Figure 3.11). The monoclonal

antibodies BE12 and AG4 failed to immunoprecipitate any detectable PrP^c from the N2a cell extracts, however, the monoclonal antibody AH6 showed promise with a small amount of PrP^c detectable. The monoclonal antibodies BE12 and AG4 recognise epitopes close to the N-terminus of PrP, and as a result they fail to recognise truncated murine PrP^c on Western blots (J. Manser, Personal communication). This may explain why they failed to recognise the PrP^c observed from the N2a cells, as this PrP^c appeared to be of a truncated form (Figure 3.10). In contrast AH6 recognises an epitope that is further from the N-terminus of PrP, and has been shown to react with truncated murine PrP^c on Western blots (J. Manser, Personal communication). Interestingly, AH6 showed a degree of reactivity with the PrP^c isolated from the N2a cells in this study, indicating that it may be a suitable antibody for immunoprecipitation studies (Figure 3.10). However, the monoclonal antibodies BE12 and AG4 may have shown the same reactivity as AH6 if a non-truncated form of PrP^c were present in the N2a cell extracts during these experiments, and as such their use as precipitating antibodies in this system cannot be ruled out. The presence of the light (LC) and heavy chains (HC) of the precipitating antibodies on the Western blots proved to be a problem. This was further complicated by apparent changes in the molecular weight of the endogenous PrP^c isolated from the N2a cells during the monoclonal antibody analysis, which appeared to result in the presence of a truncated form of PrP^c (approximately 20 kDa) (Figures 3.10 & 3.11). It is not clear why the PrP^c isolated from the N2a cell extracts with these monoclonal antibodies appeared to be truncated, but it could be due to cellular changes within the N2a cells in culture or it could be due to proteolytic activity.

It was noted that the pre-adsorption of the PrP^{sc} samples with protein A sepharose resulted in the removal of PrP^{sc} (Figure 3.2). This is likely to be due to non-specific interaction between the PrP^{sc} and the sepharose matrix, as such this pre-adsorption step was omitted in further immunoprecipitations. Protein A sepharose had already been shown to be a successful matrix in this study and the use of a protein G sepharose matrix was examined. The results showed that the use of a protein G sepharose matrix for the immunoprecipitations considerably improved the

recovery of PrP^c and as a result all subsequent immunoprecipitations were performed with protein G (Figure 3.5).

The same methods used for the immunoprecipitation of PrP^c from the murine N2a cells also proved to be successful in isolating PrP^c from murine brain tissue. PrP^c was readily detectable in wild-type mice brain extracts, however, PrP null mice brain extracts showed no detectable PrP^c (Figure 3.7).

3.3.2 Endogenous PrP^c

The methods developed for the murine N2a cells were successful at isolating endogenous PrP^c from the ovine cell cultures sA80BR and IS120Cer (Figures 3.8 & 3.9). Only one distinct PrP^c glycoform was detected from the IS120Cer cell extracts produced with Mem-per, due to its estimated size of approximately 33 kDa it is likely that this is the di-glycosylated band. However, as discussed previously, the lack of detection of the mono- and un-glycosylated bands could be due to the lower levels of PrP^c observed in the ovine cell culture samples. These results indicate that the ovine sA80BR and IS120Cer cell cultures both express endogenous PrP^c, albeit at a lower level than that observed with the murine N2a cells. The Icelandic liver derived cell culture IS120Liv showed no detectable endogenous PrP^c (Figure 3.9). However, this result was not unexpected as PrP^c is normally expressed at far lower levels in sheep liver than in brain (Caughey *et al.*, 1988, Horiuchi *et al.*, 1995, Moudjou *et al.*, 2001). The immunoprecipitation system developed in these studies was therefore capable of isolating PrP^c from a variety of mammalian cell cultures and was adaptable to isolate endogenous PrP^c from murine tissue samples.

The PrP^c and PrP^{sc} immunoprecipitated samples appeared to be missing a PrP glycoform following immunoprecipitation (Figures 3.2 & 3.3). It is not clear if the same glycoform was absent in both PrP^c and PrP^{sc}, although it appears that the mono-glycosylated form was missing from the PrP^{sc} samples, whilst the un-glycosylated from appears to be absent in the PrP^c samples (Figure 3.3). The observation that one glycoform is undetectable after the immunoprecipitation would indicate that either the precipitating antibody is unable to bind to its epitope in that glycoform, or that the missing PrP glycoform has not been extracted from the cell lysates. It is likely that in the PrP^{sc} samples the mono-glycosylated forms are not

precipitated by the 1A8 antibody, thus explaining why these glycoform bands are absent in the Western blots. As the antibody is capable of precipitating di-glycosylated PrP, there is either a conformational change between mono- and di-glycosylated PrP or these isoforms are differentially integrated into the membrane and are therefore extracted with different efficiencies. In contrast, the un-glycosylated band appears to be missing from the endogenous PrP^c due to a failure to isolate a sufficient quantity of total PrP^c from these samples. This is supported by the successful extraction of all three glycoforms of endogenous PrP^c from N2a cell extracts prepared with Mem-per, which is an extraction reagent specific for membrane proteins. It is possible that the missing glycoform band observed in the M-per samples was likely to be due to a failure to isolate that glycoform from the cell lysates. This may be due to the un-glycosylated form of PrP^c being strongly membrane associated/attached. The un-glycosylated band was consistently observed at lower levels than the di- and mono-glycosylated isoforms in the murine N2a cell extracts, therefore the intensity of the lower band may be greater in the Mem-per samples as more PrP^c is present overall.

There is evidence for the existence of truncated forms of PrP^c in cell culture and previous studies at the NPU have noted the presence of truncated PrP^c in murine N2a cells (Angie Chong, Personal communication). Other studies have revealed the presence of N-terminally truncated forms of PrP^c. Harris *et al.* (1993) showed that chicken PrP^c expressed in murine N2a cells was truncated within its N-terminal region at a point distal to the proline/glycine-rich repeat region. Lehmann & Harris (1996) showed the presence of N-terminally truncated forms of PrP in transfected Chinese hamster ovary (CHO) cells. In addition, studies by Chen, Teplow, Parchi *et al.* (1995) showed that truncated PrP^c is present in normal and prion infected human brain. Furthermore, Jimenez-Huete *et al.* (1998) revealed the presence of amino-truncated PrP^c in human brain with estimated molecular weights of 21-22 and 18 kDa.

The apparently truncated PrP^c isolated from the N2a cell extracts in the studies in this thesis was located at a molecular weight similar to the precipitating antibody light chain on Western blots, which meant that it was difficult to visualise the PrP^c. To overcome this problem the monoclonal antibodies were coupled

permanently to an amino-link matrix, which enabled the PrP^c antigen to be released from the immunocomplex without releasing the antibody heavy and light chains. It was hoped that this would have left the antibody coupled to the matrix following heat denaturation, and would enable the antibody/matrix to be re-used up to twenty times, therefore cutting the costs of the immunoprecipitation procedure. However, it appeared that although the monoclonal antibodies were successfully coupled to the amino-link matrix, the coupling procedure may have caused an alteration in the antibody structure, resulting in it being unable to bind to PrP^c (Figure 3.12).

3.3.3 Conclusions

In summary, during this study techniques for the extraction, isolation and detection of endogenous PrP^c, from ovine and murine cell extracts and murine tissue have been successfully developed. Previous studies had detected endogenous PrP^c from approximately 2 x 10^7 N2a cells, in this study endogenous PrP^c has been successfully detected from cell extracts prepared from approximately 2 x 10^5 cells. Enhancements made to this system, such as, the use of a protein G sepharose matrix and a novel buffering system have further improved the recovery of endogenous PrP^c. The cell lysis and immunoprecipitation techniques developed here were then used for the extraction of recombinant PrP^c from ovine and murine cell cultures transiently transfected with ovine PrP mini-gene constructs (Chapter 4). It was expected that the level of PrP^c extracted with this system would be sufficient to allow the detection of recombinant PrP^c expression in transiently transfected cells. The level of PrP^c isolated from the cultured cells with the present system was comparable to that achieved by other researchers from cultured neuronal cells using similar methods (Lehmann & Harris, 1996, Parizek, Roeckl, Weber *et al.*, 2001, Winklhofer *et al.*, 2003). Therefore, it was anticipated that the immunoprecipitation system developed during this study would successfully isolate recombinant PrP^c from transiently transfected cell cultures. Finally, it was expected that the techniques would prove to be important tools in the isolation of PrP in other research projects. In particular this system lends itself to the extraction of PrP from samples that may contain only low levels of PrP that would be undetectable without the use of the sample concentration step that immunoprecipitation provides.

Chapter 4: Control of ovine PrP gene expression, a role for alternative polyadenylation?

4.1 Introduction

Work described in Chapter 3 of this thesis resulted in the development of methods for the specific isolation and detection of endogenous PrP^c from ovine cell lines and from ovine tissue samples using the immunoprecipitation technique. These methods were applied in this chapter to the isolation and detection of recombinant PrP expressed by a series of ovine PrP mini-gene constructs. Polymorphisms within the open reading frame of the PrP gene do not completely explain why sheep of some genotypes are more susceptible than others to scrapie infection, as animals of different breeds with identical genotypes show variation in susceptibility to disease (Hunter, 1997, Hunter *et al.*, 1994a). It is hypothesised that other regions of the ovine PrP gene may influence scrapie susceptibility, for example, untranslated regions of the mRNA may regulate the translation of the PrP^c protein. Two mRNA transcripts of PrP have been isolated from sheep tissues, a 2.1 kb and a 4.6 kb species, in addition, a third 3.3 kb transcript may exist in peripheral tissues (Goldmann *et al.*, 1999). These different mRNA transcripts are the result of alternative polyadenylation of the PrP gene (Goldmann *et al.*, 1999, Horiuchi *et al.*, 1995, Hunter *et al.*, 1994c). The shorter 2.1 kb transcript lacks several features which are present in the 4.6 kb transcript, and these include sequences that resemble instability motifs, repetitive sequences and a highly conserved region (Goldmann *et al.*, 1999). It is known that the relative levels of PrP^c protein and PrP mRNA differ in certain tissues, suggesting that PrP gene expression may be regulated in a post-transcriptional manner, which could be linked to alternative polyadenylation (Ford *et al.*, 2002, Goldmann *et al.*, 1999, Horiuchi *et al.*, 1995). It is possible that alternative polyadenylation could be involved in the tissue-specific expression of the PrP gene, as the two PrP mRNA transcripts are found at different levels in different tissue-types (Goldmann *et al.*, 1999). The tissue specific regulation of PrP expression could involve the absence of tissue-specific factors from certain ovine tissues. Alternatively, both mRNA

transcripts could be produced in all ovine tissue types, but one may be degraded more quickly than the other due to the presence of instability motifs.

Studies by Marshall (2000) found evidence that the sequence between 2000-2700 bp in the ovine PrP 3'UTR has an inhibitory role in CAT reporter gene expression. It was hypothesised that sequences within this region act as binding sites for RNA binding proteins, which may have an inhibitory effect on the translation of mRNA transcripts containing this sequence (Marshall, 2000). The 2.1 kb transcript is polyadenylated at position 1546, therefore it lacks the putative inhibitory region, and perhaps explains why a PrP construct with a full length 3'UTR was expressed at lower levels than those containing a shorter 3'UTR (Goldmann *et al.*, 1999, Marshall, 2000). However, it is possible that these observations were an artifact of a recombinant mRNA, generated by the combination of the CAT reporter gene and PrP 3'UTR sequences. These cell culture results were also in contradiction to the *in vivo* observations that sheep brain (4.6 kb mRNA) contains a much higher content of PrP^c than peripheral tissues (4.6 kb & 2.1 kb mRNAs). Furthermore, the analysis of subtle changes in RNA levels can be complicated by the longevity of the CAT reporter protein, which has a half-life of approximately 50 hours in mammalian cells. In order to avoid these problems, PrP^c was itself used as a reporter for the action of the 3'UTR.

The work described in this chapter has investigated the influence of alternative polyadenylation on the expression of the ovine PrP gene. A series of five PrP mini-gene constructs (plasmids pConstruct1-pConstruct5) were produced (Figure 4.1) and placed under the control of a 0.5 kb ovine PrP gene promoter, which had previously been shown to be effective at promoting the expression of a CAT reporter gene (Marshall, 2000, O'Neill *et al.*, 2003). The constructs contain the complete ovine PrP gene exon III, which consists of a short 5'UTR sequence, the complete ovine PrP gene ORF, and the 3'UTR. The constructs differ only in the polyadenylation signals located within the 3'UTR (Figure 4.1).

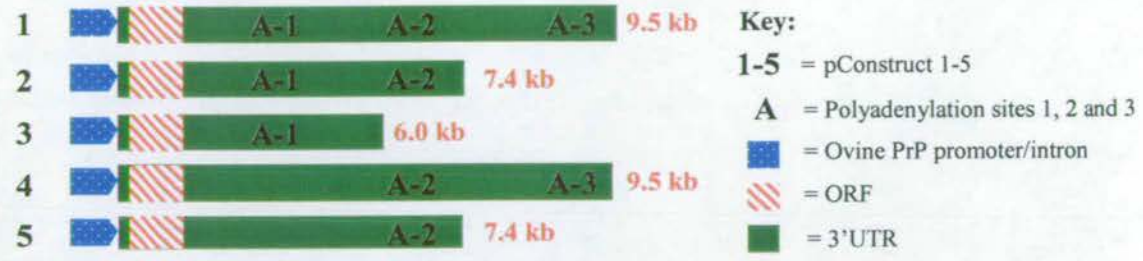


Figure 4.1 *The ovine PrP mini-gene constructs (pConstruct1-pConstruct5)*

Ovine PrP mini-gene constructs 1-5. Each construct contains a 0.5 kb Cheviot sheep PrP gene promoter fragment, a 700 bp intron sequence and the Suffolk sheep PrP gene exon III, which contains a short 5' UTR sequence, the full ovine PrP gene ORF and a 3'UTR sequence. The contructs differ only in the length of the 3'UTR sequence and the available polyadenylation signals.

The ovine PrP mini-gene constructs were used to investigate the influence of the 3'UTR on PrP^c expression following transient transfection of ovine and murine cell cultures (Sections 2.5.1-2.5.3). Immunoprecipitation has been shown to be a successful method of purifying PrP^c from N2a cell extracts (Marshall, 2000). In addition, work carried out during this study successfully developed techniques for the efficient extraction of endogenous PrP^c from murine N2a cells, ovine sA80BR and IS120Cer cells, and murine brain tissue (Chapter 3). These techniques were applied to the immunoprecipitation of recombinant PrP^c from the ovine and murine cell cultures transiently transfected with the ovine PrP mini-gene constructs.

Marshall (2000) modified the sequence of the ovine PrP gene ORF to carry a hamster specific epitope (valine to methionine substitution at codon 115) that was specifically recognised by the 3F4 antibody. The use of this epitope was believed to be a suitable method to enable the detection of recombinant PrP^c against a background of endogenous PrP^c (Kascsak, Rubenstein, Merz *et al.*, 1987, Marshall, 2000). The results of transient transfections showed that the 3F4 antibody cross reacted with endogenous murine and ovine PrP^c and this was also shown by Baron, Betemps, Groschup *et al.* (1999). The 3F4 epitope was therefore unsuitable for the detection of recombinant PrP^c against a background of endogenous PrP^c. Another

tagging epitope (3X FLAG™, Sigma, UK) was chosen for this study. Previous work by Telling, Tremblay, Torchia *et al.* (1997) had shown that the incorporation of a single FLAG™ epitope at the N-terminus of the mature murine PrP^c protein had no effect on its normal cellular processing. For the present study the 3X FLAG™ epitope was inserted C-terminal of the signal peptide. A *Xma*I restriction site in the ORF (codons 42-43) was used for the insertion of the 3XFLAG™ coding DNA fragment. Therefore, the 3XFLAG™ epitope will be positioned between amino acid codons 43 and 44 of ovine PrP^c. By using PrP mini-gene expression constructs with differing combinations of the available polyadenylation signals, the role of these signals in PrP gene expression could be investigated, and it was expected that the studies would provide further insights into the role of the 3'UTR inhibitory region (Goldmann *et al.*, 1999, Marshall, 2000). By expressing these constructs in a range of ovine cell types, including cells derived from animals of differing PrP genotypes, an attempt was made to investigate the role of alternative polyadenylation in the tissue-specific expression of the PrP gene.

4.2 Results

4.2.1 Cloning of the 3XFLAG™ tagged ovine PrP mini-gene constructs

4.2.1.1 Generation of a 3XFLAG™ DNA sequence for cloning into exon III of the ovine PrP gene

The expression vector p3XFLAG-CMV-8™ (Sigma, UK) was used as a source of the 3XFLAG™ DNA sequence. PCR primers (Flag Forward and Flag Reverse, Table 2.1) were designed to amplify the 3XFLAG™ DNA sequence of 66 bp from the p3XFLAG-CMV-8™ vector. The primers were designed to include *Xma*I restriction endonuclease sites at either end (Figure 4.2, red text). The *Xma*I sites were subsequently used to clone the 3XFLAG™ DNA into an *Xma*I site in the ovine PrP gene ORF. In order to maintain the reading frame with both the ovine PrP gene and the 3XFLAG™ DNA sequence, an additional base (A) was added to the primer at the

5'end of the 3XFLAG™ sequence (Flag Forward primer) and two additional bases (TA) were added at the 3'end (Flag Reverse primer) (Figure 4.2, green text).

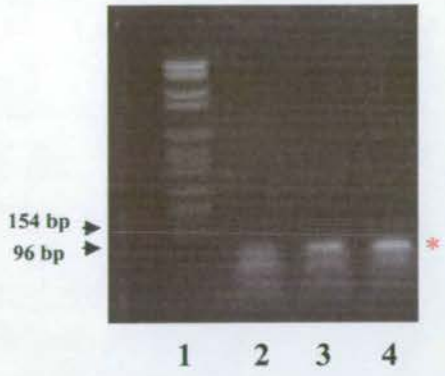
Flag forward primer	5' TCTCTACCCGGGAGACTACAAAGAC' 3
Flag reverse primer	5' CTAAATCCCGGGTACTTGTCATCGTC' 3

KEY:
Leader sequence for *Xma*I binding
***Xma*I restriction enzyme binding site**
Additional bases added to maintain reading frame
3XFLAG™ DNA sequence

Figure 4.2 *Primers used for the PCR amplification of the modified 3XFLAG™ DNA sequence from the plasmid p3XFLAG-CMV-8™*

The primers used for the amplification of the 3XFLAG™ DNA sequence were modified to incorporate additional bases (green bases) into the 3XFLAG™ sequence in order to maintain reading frame with the ovine PrP ORF and the 3XFLAG™ epitope and *Xma*I restriction sites for use in DNA cloning.

To reduce the risk of erroneous amplification of the p3XFLAG-CMV-8™ vector DNA, the vector was first digested with the restriction endonucleases *Spe*I and *Age*I (Section 2.2.1) producing a 1317 bp fragment containing the 3XFLAG™ DNA sequence and a 3446 bp plasmid DNA fragment. The 3446 and 1317 bp fragments were separated by gel electrophoresis (Section 2.2.6), the 1317 bp fragment was isolated by gel extraction (Section 2.2.7) and used as template DNA for PCR amplification (Section 2.2.14) of the 3XFLAG™ DNA sequence. Following PCR amplification, the presence of the 93 bp 3XFLAG™ DNA sequence (66 bp 3XFLAG™ DNA, 3 bp additional bases added to maintain frame 12 bp *Xma*I site DNA and 12 bp additional bases (six bases 5' and six bases 3') added to ensure that the restriction enzyme *Xma*I would be able to bind to the DNA fragment) was confirmed by agarose gel electrophoresis (Section 2.2.6) of the PCR product (Figure 4.3).

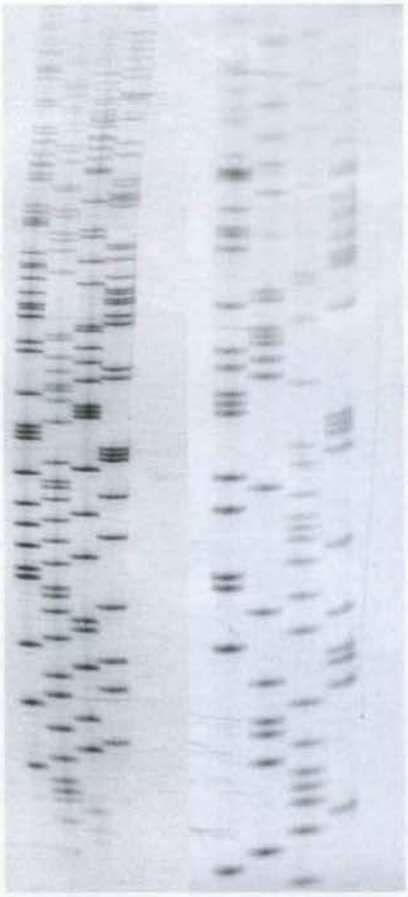


KEY: Lane 1, Molecular weight marker VI (Roche, UK); Lane 2, PCR product negative for 93 bp 3XFLAG™ DNA fragment; Lanes 3 + 4, PCR products positive for 93 bp 3XFLAG™ DNA fragment. * indicates location of 93 bp 3XFLAG™ DNA fragment.

Figure 4.3 *Successful amplification of the 93 bp 3XFLAG™ DNA sequence by PCR from vector p3XFLAG-CMV-8™ analysed by gel electrophoresis*

The amplification of the 3XFLAG™ DNA sequence was confirmed by gel electrophoresis of the PCR reaction product which showed the presence of an approximately 90-100 bp DNA fragment, corresponding to the 93 bp modified 3XFLAG™ DNA sequence.

The PCR amplified 3XFLAG™ DNA sequence was isolated by gel extraction (2.2.7), and ligated into the vector pGEM-T Easy™ (Promega, UK) to produce the vector pFLAG. The incorporation of the modified 3XFLAG™ DNA sequence into pFLAG was confirmed by Sanger sequencing (Section 2.3) using Flag forward and Flag reverse (Table 2.1) PCR primers (Figure 4.4), the final sequence of the 3XFLAG™ PCR fragment within the vector pFLAG is shown in Figure 4.5.



KEY: G = guanine, A = adenine, T = thymine, C = cytosine

Figure 4.4 *Sanger sequencing gel of pFLAG mini-prep DNA using the Flag forward & Flag reverse primers*

Sanger sequencing gel shows the correct sequence for the modified 3XFLAG™ DNA fragment within the vector pFLAG, including the *Xma*I restriction site and the additional bases included to maintain reading frame. It also shows the correct sequence for the 3XFLAG™ DNA.

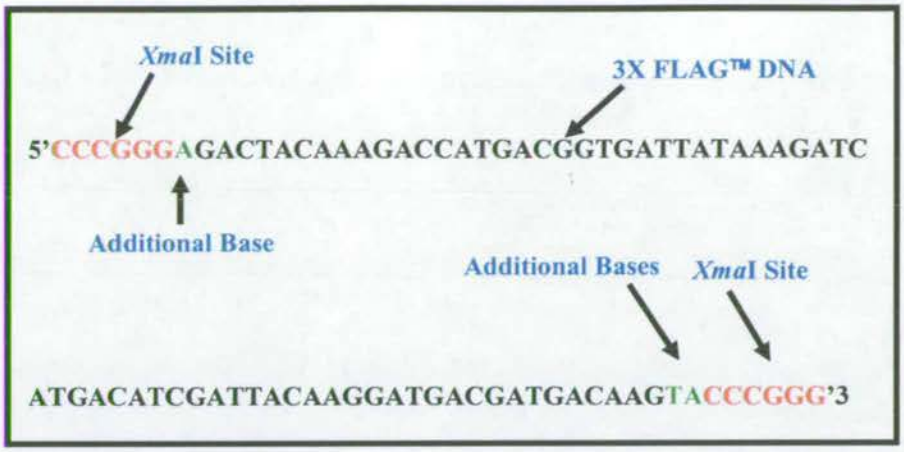


Figure 4.5 *Final sequence of the 3XFLAG™ DNA PCR fragment (top strand) as confirmed by manual sequencing of the vector pFLAG*

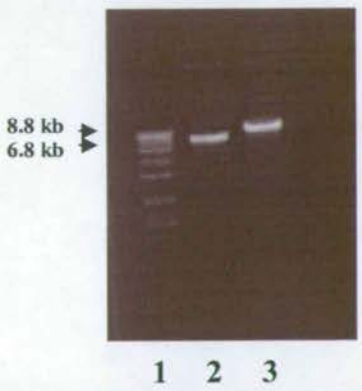
Diagram shows the modified 3XFLAG™ DNA sequence in the 5' – 3' direction. The positions of the additional bases (green text) and the *Xma*I restriction sites (red text) and the 3XFLAG™ sequence are indicated.

The modified 3XFLAG™ DNA sequence was removed by digestion of pFLAG with *Xma*I (Section 2.2.1), producing a 76 bp fragment which was isolated by gel extraction (Sections 2.2.6 & 2.2.7) ready for cloning into the *Xma*I site in exon III of the ovine PrP gene (Section 4.2.1.4).

4.2.1.2 Insertion of the ovine PrP gene exon III into the vector pBluescript-SK to produce the plasmid pSK7

In order to produce a plasmid which contained the ovine PrP gene exon III with all three polyadenylation sites the plasmid pBluescript-SK (Stratagene, UK) was used. pBluescript-SK was digested with the restriction endonucleases *EcoR*I and *Spe*I (Section 2.2.1) within its multi-cloning site (Figure 4.7, Page 141). This resulted in the removal of a *Xma*I restriction site from the multi-cloning site of the plasmid, which allowed the 3XFLAG™ DNA sequence to be inserted into a unique *Xma*I site within the ovine PrP gene exon III. The ovine PrP gene exon III was then removed

from the plasmid p71 (Wilfred Goldmann, NPU) with the *Eco*RI and *Spe*I restriction enzymes (Section 2.2.1). The ovine PrP gene exon III *EcoR*I site lies at position 21532 bp, whilst the *Spe*I site lies at position 27363 bp, using the Suffolk PrP gene numbering (GenBank accession number = U67922). The digestion resulted in the removal of a short section of DNA at the 3'end of exon III, leaving all three polyadenylation sites intact and producing an exon III fragment of approximately 6 kb (Figure 4.7). The *EcoRI/Spe*I exon III fragment was ligated (Section 2.2.4) into pBluescript-SK to produce the plasmid pSK7 (Figure 4.7). The presence of the ovine PrP gene exon III fragment was confirmed by digestion of pSK7 with *Xma*I (Section 2.2.1) and analysis by gel electrophoresis (Section 2.2.6). The *Xma*I restriction site is only present in the ovine PrP gene exon III fragment and is not present in the plasmid pBluescript-SK once digested with *Eco*RI and *Spe*I, therefore, only clones which contained the exon III fragment would be linearised (Lane 3, Figure 4.6).



KEY: Lane 1, Molecular weight marker VII (Roche, UK); Lane 2, *Xma*I digest & linearisation of pG3 DNA; Lane 3, *Xma*I digest & linearisation of pSK7 DNA.

Figure 4.6 *Confirmation of the presence of the 3XFLAG™ DNA sequence in the plasmids pSK7 & pG3 by digestion with Xma*I

Presence of 3XFLAG™ DNA sequence was confirmed by digestion with the restriction enzyme *Xma*I which cleaved the plasmids pSK7 (8.8 kb) and pG3 at one site only, confirming that the 3XFLAG™ DNA sequence (containing a unique *Xma*I site) was successfully inserted into the plasmids which did not contain an *Xma*I site.

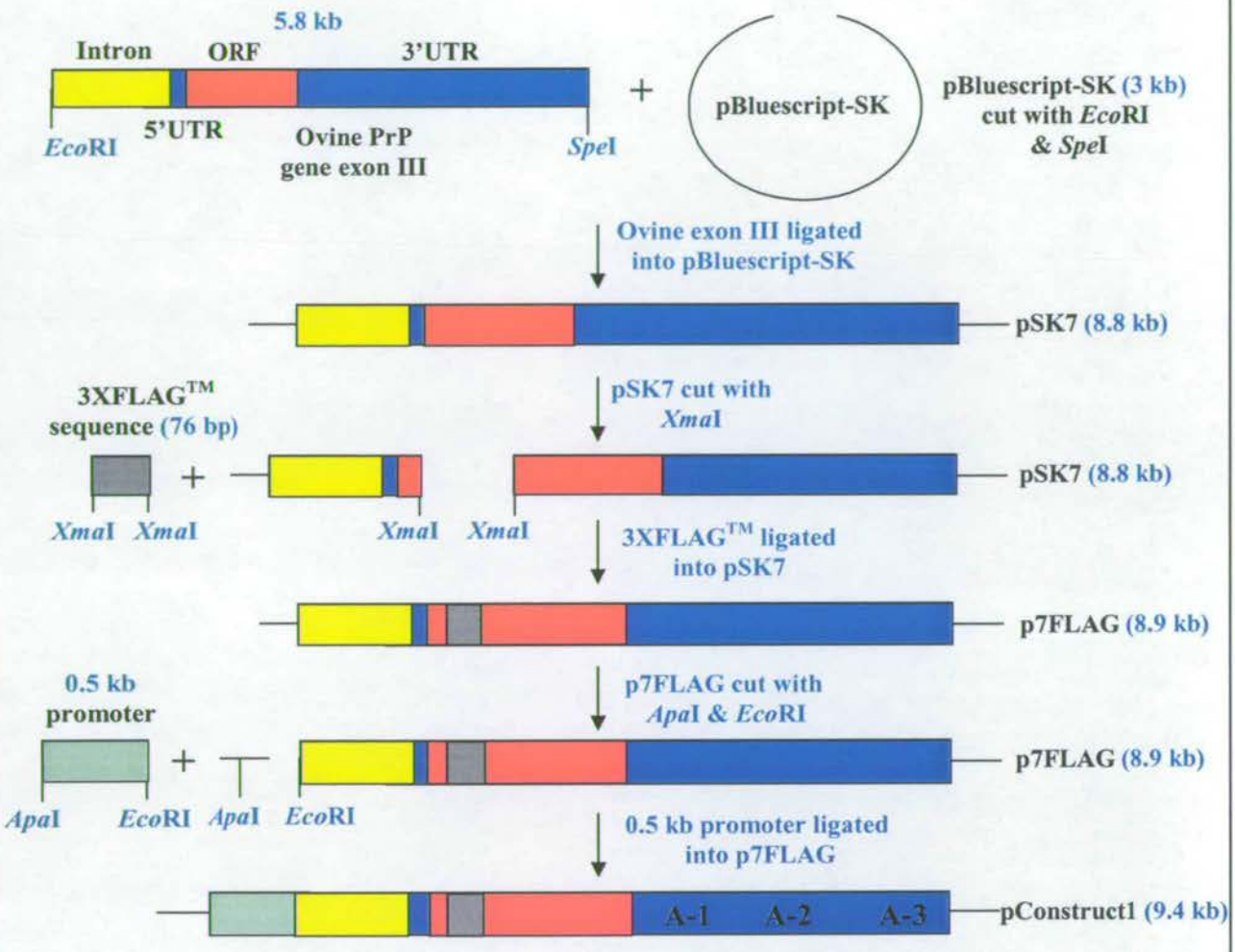


Figure 4.7 *Cloning strategy for ovine PrP mini-gene Construct 1 (pConstruct1)*

The Suffolk sheep (Genbank U67922) PrP gene exon III, including the ovine PrP gene ORF, a short 5'UTR, the 3'UTR (cleaved at the *Spe*I restriction site) and a 700 bp intron sequence (5') (total fragment size of 5.8 kb) was cloned into pBluescript-SK (3 kb) using *Eco*RI & *Spe*I restriction sites to produce pSK7 (total plasmid size of 8.8 kb). The modified 3XFLAG™ sequence (76 bp) was cloned into a unique *Xma*I restriction site within the ovine PrP gene ORF in pSK7 to produce p7FLAG (8.9 kb). The 0.5 kb Cheviot PrP promoter fragment was cloned 5' of the ovine PrP gene exon III fragment in p7FLAG using *Apa*I & *Eco*RI restriction sites to produce pConstruct1 (approximately 9.4 kb). Polyadenylation sites are marked A-1 to A-3.

4.2.1.3 *Insertion of the ovine PrP gene exon III into the vector pGEM to produce the plasmid pG3*

In order to produce a plasmid which contained the ovine PrP gene exon III with only two polyadenylation sites, numbers 1 and 2, the plasmid pGEM-7Zf(+)

(Promega, UK) was cut with the restriction endonucleases *EcoR*I and *Cla*I (Section 2.2.1) within its multi-cloning site (Figure 4.8). This resulted in the linearisation of the plasmid and in the loss of a X*ma*I site from this vector, which aided in the later cloning of the 3XFLAG™ DNA sequence (Figure 4.8). The ovine PrP gene exon III fragment was removed from the plasmid pGEM.3kb (Elaine Marshall, NPU) with the *Eco*RI & *Cla*I restriction enzymes (Section 2.2.1). The ovine PrP gene exon III *EcoR*I site lies at position 21532 bp, whilst the *Cla*I site lies at position 25297 bp, using the Suffolk PrP gene numbering (GenBank accession number = U67922). This digestion resulted in the removal of a section at the 3'end of exon III, including the removal of the third polyadenylation site, producing an exon III fragment of approximately 4 kb. The *EcoRI*/*Cla*I exon III fragment was ligated into pGEM-7Zf(+), producing the plasmid pG3 (Section 2.2.4). The presence of the ovine PrP gene exon III fragment was confirmed by digestion of pG3 with *Xma*I (Section 2.2.1) and analysis by gel electrophoresis (Section 2.2.6). The *Xma*I site is only present in the ovine PrP gene exon III fragment and is not present in the plasmid pGEM-7Zf(+) once digested with *Eco*RI and *Cla*I, therefore, only clones which contained the exon III fragment would be linearised (Lane 2, Figure 4.6, Page 140).

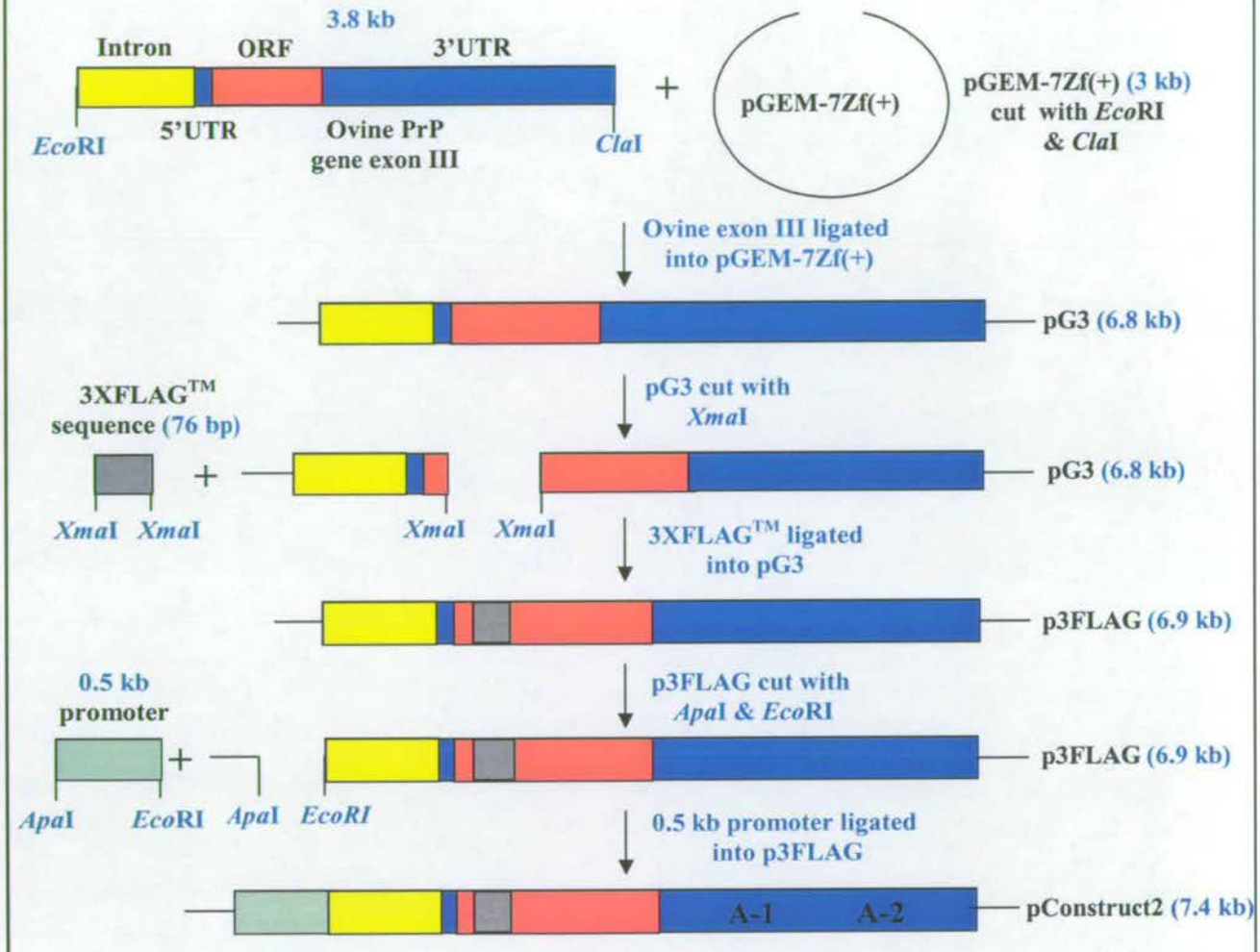


Figure 4.8 *Cloning strategy for ovine PrP mini-gene Construct 2 (pConstruct2)*

The Suffolk sheep (Genbank U67922) PrP gene exon III, including the ovine PrP gene ORF, a short 5'UTR, the 3'UTR (cleaved at the *Cla*I restriction site) and a 700 bp intron sequence (5') (total fragment size of 3.8 kb) was cloned into pGEM-7Zf(+) (3 kb) using *Eco*RI & ClaI restriction sites to produce pG3 (6.8 kb). The modified 3XFLAG™ sequence (76 bp) was cloned into a unique *Xma*I restriction site within the ovine PrP gene ORF in pG3 to produce p3FLAG (6.9 kb). The 0.5 kb Cheviot PrP promoter fragment was cloned 5' of the ovine PrP gene exon III fragment in p3FLAG using *Apa*I & *Eco*RI restriction sites to produce pConstruct2 (7.4 kb). Polyadenylation sites are marked A-1 to A-3.

4.2.1.4 Insertion of the 3XFLAG™ DNA sequence into the ovine PrP gene exon III within the plasmids pSK7 & pG3

The plasmids pSK7 (Section 4.2.1.2) & pG3 (Section 4.2.1.3) were cut at the unique *Xma*I restriction site within the ovine PrP gene exon III (Section 2.2.1) and

the 3XFLAG™ DNA sequence fragment, flanked by *Xma*I restriction sites (Section 4.2.1.1) was ligated into the linearised plasmids (Section 2.2.4), producing the plasmids p7FLAG and p3FLAG, respectively (Figures 4.7 & 4.8, Pages 141 & 143).

4.2.1.5 Sequencing of p7FLAG and p3FLAG DNA

The correct sequence of the 3XFLAG™ DNA within the ovine PrP gene exon III in the plasmids p7FLAG and p3FLAG was confirmed by Sanger sequencing (Section 2.3) using the Flag Reverse primer (Table 2.1) (Figure 4.9). The full sequence is shown in Figure 4.10 (Page 146).

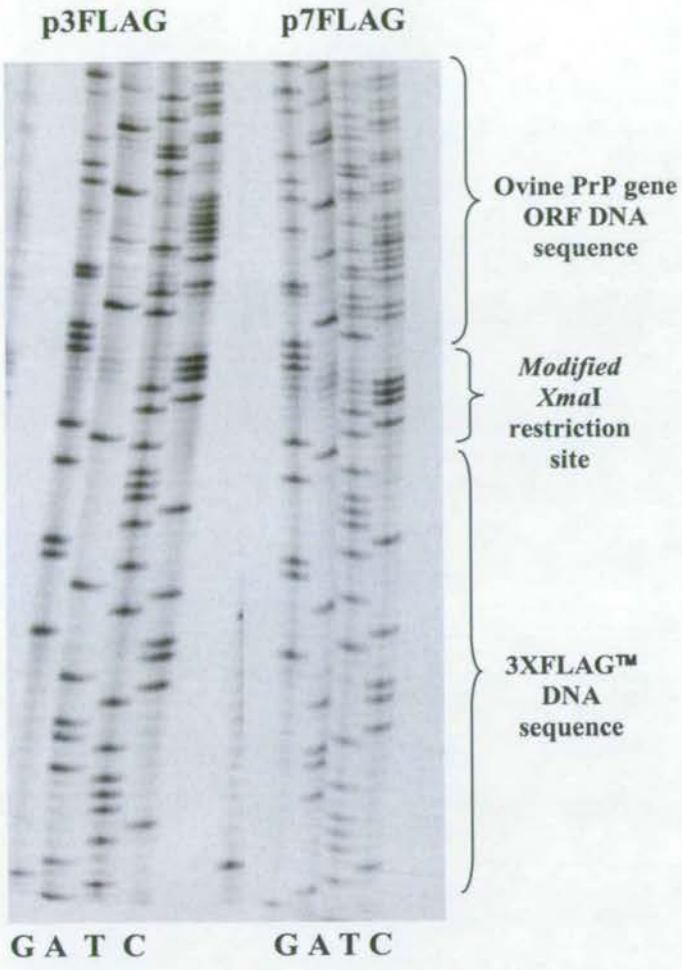


Figure 4.9 *Sanger sequencing gel of p3FLAG & p7FLAG plasmid DNA using the Flag reverse primer*

Sanger sequencing gel shows the correct sequence for the modified 3XFLAG™ DNA fragment within the vectors p3FLAG & p7FLAG, including the *Xma*I restriction site and the additional bases included to maintain reading frame. It also shows the correct sequence and location of the 3XFLAG™ DNA sequence within the ovine PrP gene ORF.

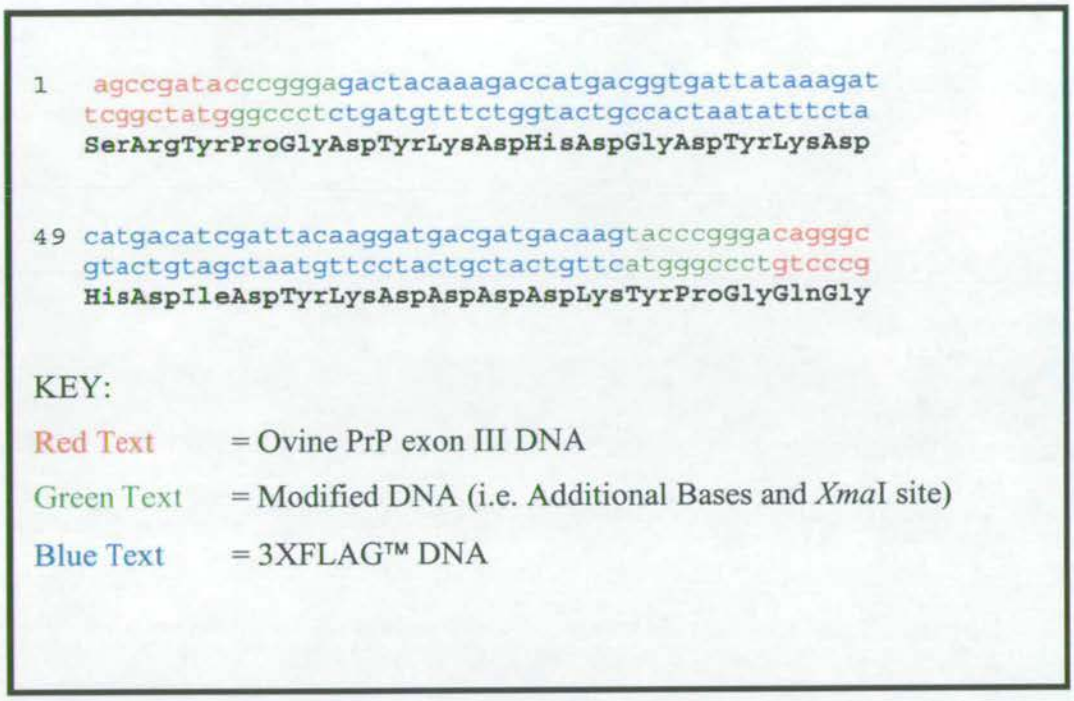


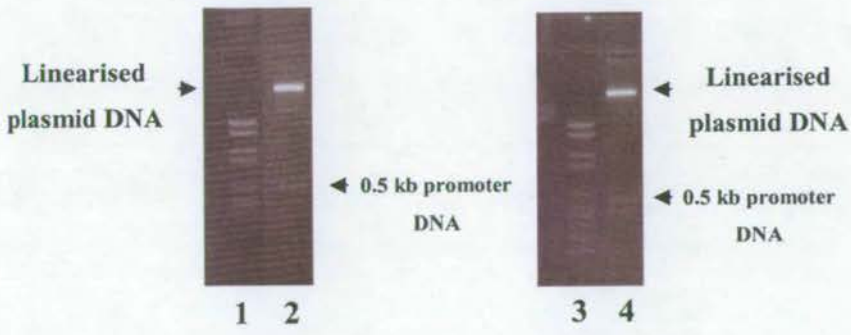
Figure 4.10 *3XFLAG™ DNA sequence within the ovine PrP exon III in the plasmids p7FLAG and p3FLAG*

Diagram shows the modified ovine PrP gene ORF including the 3XFLAG™ sequence incorporated into the unique *Xma*I restriction site. The diagram also shows how reading frame of both the 3XFLAG™ sequence and the ovine PrP gene ORF were maintained by the use of additional bases.

4.2.1.6 *Insertion of the Cheviot PrP promoter fragment into the plasmids p7FLAG and p3FLAG to produce the ovine PrP mini-gene constructs (pConstruct1 & pConstruct2)*

The 0.5 kb ovine PrP gene promoter fragment was isolated from the plasmid pNPU-110 (O'Neill *et al.*, 2003), using the restriction endonucleases *Eco*RI and *Sph*I (Section 2.2.1). The promoter fragment was then ligated (Section 2.2.4) into the plasmid pGEM-T Easy™ using the same restriction sites. The reason for cloning the 0.5 kb promoter into pGEM-T Easy™ was that it contained *Eco*RI and *Apa*I restriction sites, which would allow the insertion of the promoter into the plasmids

p7FLAG and p3FLAG, in the correct position and orientation. The 0.5 kb promoter fragment was cleaved from pGEM-T Easy™ with the restriction endonucleases *Eco*RI and *Apa*I (Section 2.2.1) and isolated by gel extraction (Sections 2.2.6 & 2.2.7). The resulting ovine PrP gene promoter fragment was ligated into linearised plasmids p7FLAG and p3FLAG using the *Eco*RI and *Apa*I restriction sites (Section 2.2.4), which positioned the promoter in the correct orientation 5' to the ovine PrP gene exon III fragment, producing the ovine PrP mini-gene Constructs 1 (pConstruct1) and 2 (pConstruct2), respectively (Figures 4.7 & 4.8, Pages 141 & 143). The presence of the ovine PrP gene promoter within these plasmids was confirmed by digestion with *Eco*RI and *Apa*I (Section 2.2.1) and analysis by gel electrophoresis (Section 2.2.6) (Figure 4.11).



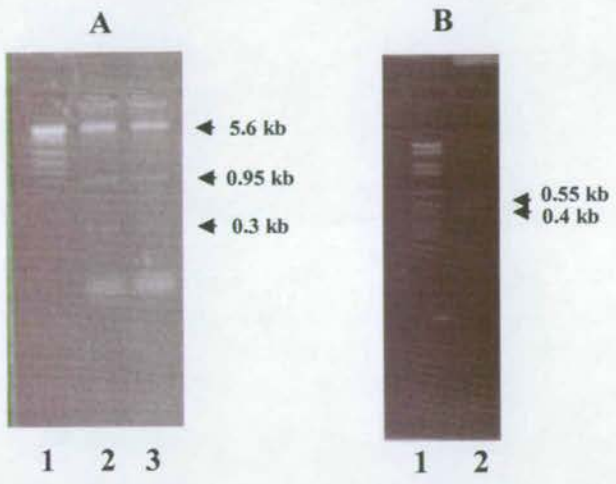
KEY: Lanes 1 & 3, Molecular weight marker VI (Roche, UK); Lane 2, pConstruct1 DNA digested with *Eco*RI & *Apa*I; Lane 4, pConstruct2 DNA digested with *Eco*RI & *Apa*I.

Figure 4.11 *Confirmation of the presence of the 0.5 kb Cheviot promoter sequence within pConstruct 1 & pConstruct2 by digestion with EcoRI & ApaI*

Figure shows the presence of the 0.5 kb Cheviot promoter DNA fragment as confirmed by gel electrophoresis of pConstruct1 and pConstruct2 DNA following digestion with *Eco*RI & *Apa*I restriction enzymes. The linearised plasmid DNA can also be clearly seen, indicating the successful digestion of the construct DNA.

4.2.1.7 Generation of the ovine PrP mini-gene Construct 3 (pConstruct3) by manipulation of the plasmid pConstruct2

pConstruct2 was digested with the restriction endonucleases *Kpn*I and *Nsi*I (Section 2.2.1), producing *Kpn*I/*Nsi*I digestion fragments of 5.6 kb, 0.95 kb and two *Nsi*I digestion fragments of 0.3 kb each (Set A, Figure 4.12).



KEY: Set A: Lane 1, Molecular weight marker VII (Roche, UK); Lanes 2 & 3, pConstruct2 DNA digested with *Kpn*I & *Nsi*I. Set B: Lane 1, Molecular weight marker VI (Roche, UK); Lane 2, 0.95 kb fragment digested with the restriction enzyme *Pst*I.

Figure 4.12 *Preparation of pConstruct3 by digestion of pConstruct2*

Figure A shows the presence of the 0.3 kb, 0.95 kb and 5.6 kb bands following digestion of pConstruct2 with *Kpn*I & *Nsi*I restriction enzymes. The 5.6 kb band was isolated by gel extraction and the 0.3 kb band was discarded. Figure B shows the presence of the 0.55 kb & 0.4 kb bands following digestion of the 0.95 kb band with the *Pst*I restriction enzyme.

The 5.6 kb and 0.95 kb fragments were separated by gel electrophoresis (Section 2.2.6) and isolated by gel extraction (Section 2.2.7), whilst the two 0.3 kb *Nsi*I digestion fragments were discarded. The 0.95 kb fragment was further digested with the restriction endonuclease *Pst*I (Section 2.2.1), producing a 0.4 kb fragment and a 0.55 kb fragment. These two fragments were further separated by gel electrophoresis (Section 2.2.6), and the 0.55 kb fragment, containing the second

polyadenylation sequence was discarded (Set B, Figure 4.12). The 0.4 kb fragment, flanked by *Pst*I and *Kpn*I sites was isolated by gel extraction (Section 2.2.7) and re-ligated into the initial 5.6 kb *Kpn*I/*Nsi*I fragment using the homologous *Pst*I/*Nsi*I restriction sites (Section 2.2.4) and the *Kpn*I restriction site to produce pConstruct3 (Figure 4.13).

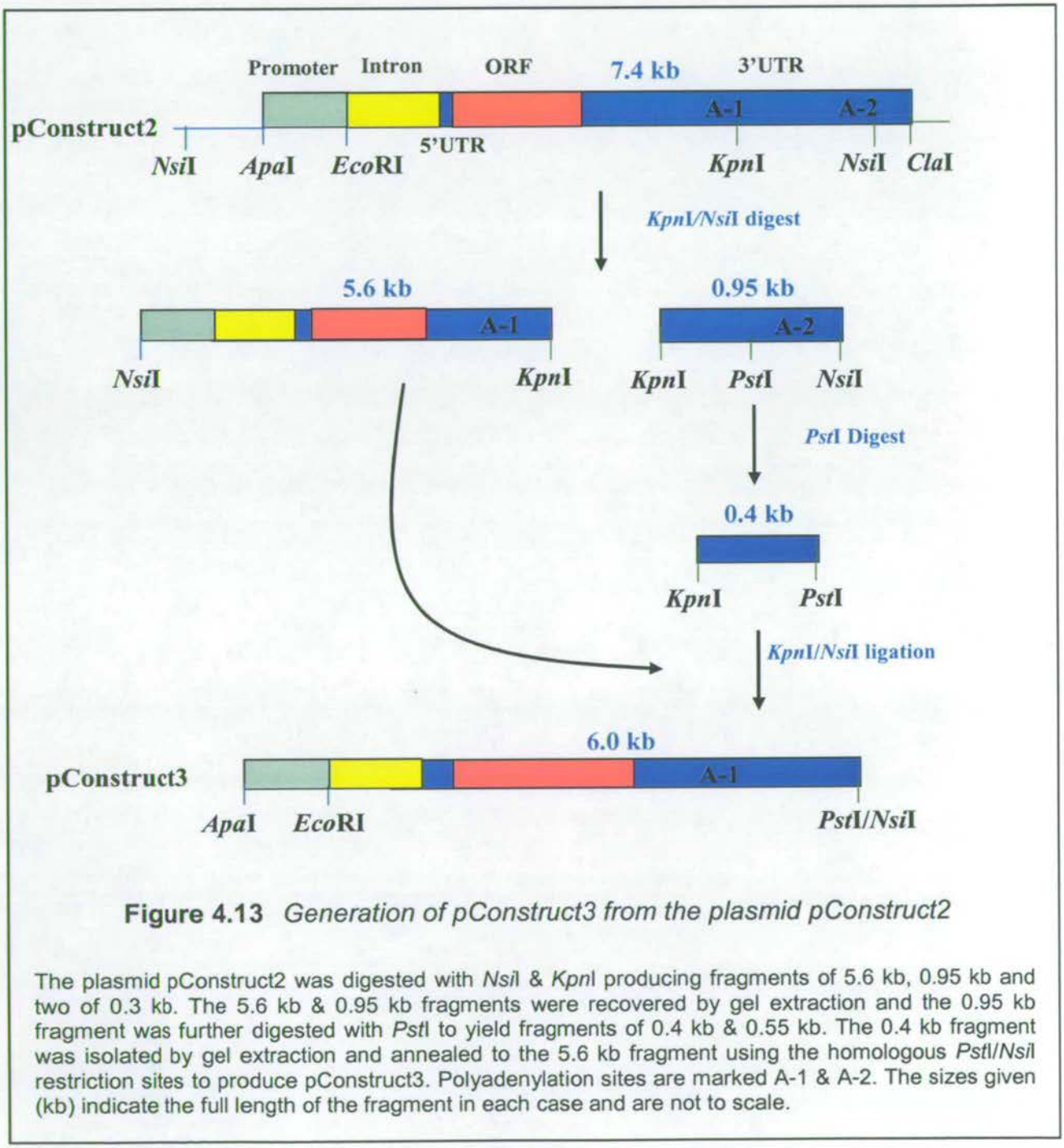
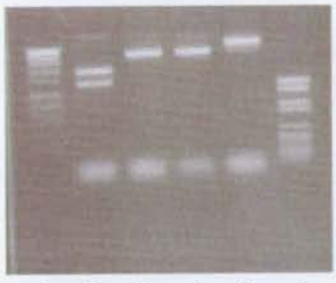


Figure 4.13 *Generation of pConstruct3 from the plasmid pConstruct2*

The plasmid pConstruct2 was digested with *Nsi*I & *Kpn*I producing fragments of 5.6 kb, 0.95 kb and two of 0.3 kb. The 5.6 kb & 0.95 kb fragments were recovered by gel extraction and the 0.95 kb fragment was further digested with *Pst*I to yield fragments of 0.4 kb & 0.55 kb. The 0.4 kb fragment was isolated by gel extraction and annealed to the 5.6 kb fragment using the homologous *Pst*I/*Nsi*I restriction sites to produce pConstruct3. Polyadenylation sites are marked A-1 & A-2. The sizes given (kb) indicate the full length of the fragment in each case and are not to scale.

The successful production of the plasmid pConstruct3 was confirmed by digesting pConstruct3 with the restriction enzymes *Xba*I, *Cla*I, *Kpn*I & *Nsi*I (Figure 4.14). Digestion with *Xba*I produced two fragments of 2.6 & 3.4 kb, consistent with a successful ligation (Figure 4.14). Digestion with *Cla*I and *Kpn*I both linearised the plasmid pConstruct3 producing a 6 kb fragment as expected, whilst digestion with *Nsi*I did not cut pConstruct3 (Figure 4.14) consistent with the *Nsi*I site being ablated following the ligation of this site with the *Pst*I site (Figure 4.13).



1 2 3 4 5 6

KEY: Set A: Lane 1, Molecular weight marker VII (Roche, UK); Lane 2, pConstruct3 DNA digested with the restriction enzyme *Xba*I; Lane 3, pConstruct3 DNA digested and linearised with the restriction enzyme ClaI; Lane 4, pConstruct3 DNA digested and linearised with the restriction enzyme *Kpn*I; Lane 5, pConstruct3 DNA digested and not linearised with the restriction enzyme *Nsi*I; Lane 6, Molecular weight marker VI (Roche, UK).

Figure 4.14 *Confirmation of the successful production of the plasmid pConstruct3 by digestion with restriction enzymes*

The successful production of pConstruct3 was confirmed by digestion of the plasmid DNA with the following restriction enzymes: *Xba*I producing two fragments of 2.6 & 3.4 kb consistent with a successful ligation, *Cla*I or *Kpn*I producing a linearised DNA fragment of 6 kb, *Nsi*I which failed to cut the plasmid consistent with the successful ablation of this site following the re-ligation of the pConstruct3 DNA via the *Pst*I/*Nsi*I restriction sites.

4.2.1.8 Ablation of polyadenylation site 1 within the plasmids pConstruct1 and pConstruct2 to produce the plasmids pConstruct4 and pConstruct5

The ovine PrP mini-gene Constructs 4 (pConstruct4, polyadenylation sites 2 & 3) and 5 (pConstruct5, polyadenylation site 2) were produced from the plasmids pConstruct1 (polyadenylation sites 1, 2 & 3) and pConstruct2 (polyadenylation sites 1 & 2), respectively. The first polyadenylation site in the ovine PrP gene exon III fragment within the plasmids pConstruct1 and pConstruct2 lies within a unique *Pac*I restriction endonuclease site, and these plasmids were linearised with *Pac*I (Section 2.2.1), leaving 3'overhangs on both ends (Figure 4.15). The 3' overhangs were then digested with the Klenow fragment of DNase I (Section 2.2.2), deleting the two bases from the first polyadenylation sequence and leaving blunt ends. The blunt ended plasmids were re-ligated (Section 2.2.4) to produce the plasmids pConstruct4 and pConstruct5 (Figure 4.15).

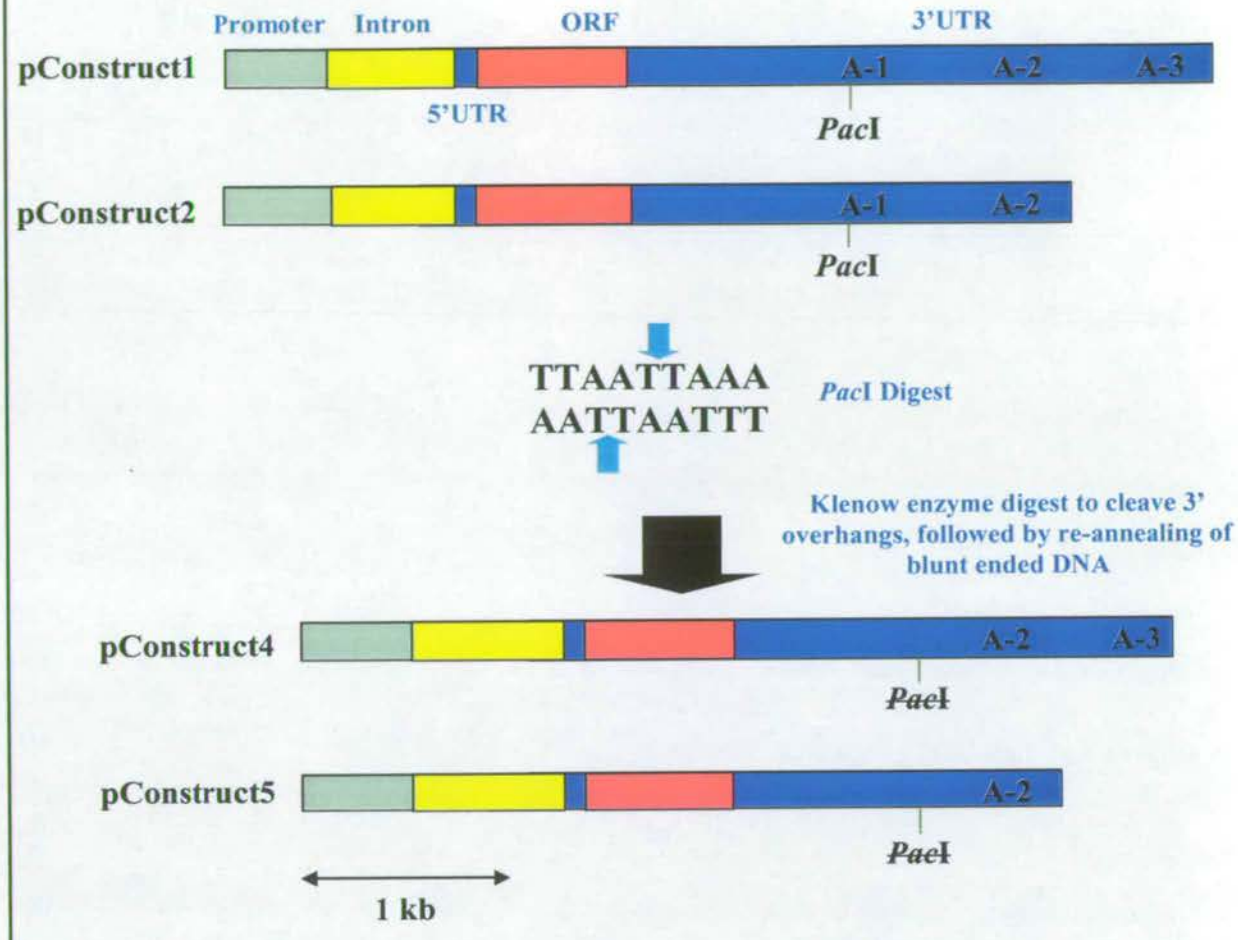
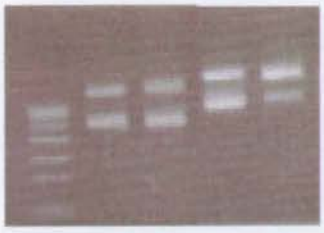


Figure 4.15 *Ablation of the first polyadenylation site within the ovine PrP exon III within the plasmids pConstruct2 & 5 to produce pConstruct4 & 5*

The first polyadenylation signal in the plasmids pConstruct1 & pConstruct2 was ablated by digestion of the plasmids at the unique *Pac*I restriction site that lies within this signal. Digestion with *Pac*I left 3' overhangs on both ends of the digested DNA, which were digested with the Klenow fragment of DNase I leaving blunt ends. The blunt ends were then annealed to produce the plasmids pConstruct4 & pConstruct5 which contained the now ablated first polyadenylation signal. Polyadenylation sites are marked A-1 to A-3. Size bar indicates approximate length of 1 kb of DNA.

The ablation of the *Pac*I restriction site within the plasmids pConstruct4 and pConstruct5 was confirmed by digestion with the *Pac*I restriction endonuclease, which did not cut the plasmids, indicating that the site had been successfully deleted (Figure 4.16). Finally, maxi-prep DNA of all five ovine PrP mini-gene constructs

(plasmids pConstruct1-pConstruct5) was produced (Section 2.2.12), ready for transient transfection into the ovine cell cultures (Section 4.2.5).



KEY: Lane 1, Molecular weight marker VII (Roche, UK); Lanes 2 & 3, pConstruct5 DNA digested and not linearised with the restriction enzyme *Pac*I; Lanes 4 & 5, pConstruct4 DNA digested and not linearised with the restriction enzyme *Pac*I.

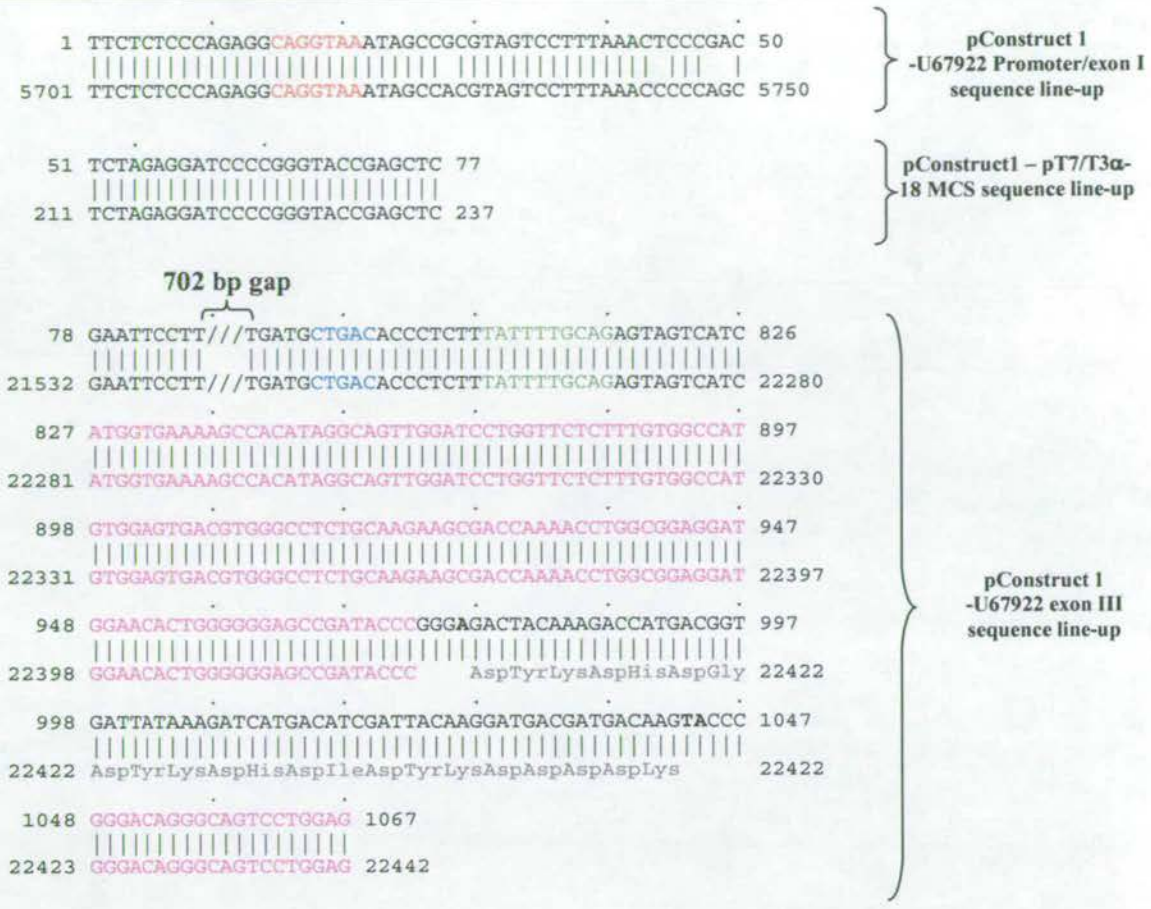
Figure 4.16 *Confirmation of the ablation of the PacI restriction site within exon III in the plasmids pConstruct4 & pConstruct5*

The successful ablation of the first polyadenylation site within the ovine PrP gene 3'UTR was confirmed by digestion of the pConstruct4 & pConstruct5 DNA with *Pac*I. The construct DNA was not cleaved by *Pac*I indicating the successful ablation of the first polyadenylation site and re-annealing of the construct DNA.

4.2.1.9 Sequencing of the plasmids pConstruct1 & pConstruct2

pConstruct1 and pConstruct2 were sequenced in order to confirm that the required elements were in place in the PrP mini-gene constructs that would allow the mini-gene RNAs to be correctly spliced and processed. pConstruct1 and pConstruct2, upon which plasmids pConstruct3-pConstruct5 were based, were sequenced by the Sanger method (Section 2.3), using a series of three ovine PrP gene promoter-specific primers (Promseq 1-3, Table 2.2). Sanger sequencing confirmed that all of the splicing elements were in place, including the 5' splice donor sequence (red text, Figure 4.17, Page 155), the 3' splice acceptor sequence (green text, Figure 4.17) and the branch-point sequence (blue text, Figure 4.17). This figure shows a line-up of the pConstruct1 sequence (1-1067 bp) with the Suffolk PrP gene sequence (5701-22422 bp, GenBank accession number = U67922). The ovine PrP mini-gene

sequence is homologous to the U67922 promoter/intron sequence, however, it does highlight the four single base changes between the Suffolk promoter sequence (U67922), and the Cheviot promoter sequence used in this study (Figure 4.17). A short 27 base segment of DNA from the multi-cloning site of the plasmid pNPU-110 (based on the original plasmid pT7/T3α-18) from which the 0.5 kb Cheviot promoter was obtained (Section 4.2.1.6), was incorporated into all of the constructs. The 27 bp sequence was lined-up against the original pT7/T3α-18 plasmid sequence (211-237 bp) with no base changes observed, and is shown in Figure 4.17. In addition, a 702 bp gap was introduced in this sequence diagram to allow the 5' end of exon III to be shown (Figure 4.17). The line-up highlights the incorporation of the 3XFLAG™ sequence into the ovine exon III DNA, and demonstrates how the frame of both the PrP ORF (magenta text, Figure 4.17), and the 3XFLAG™ epitope was maintained by the incorporation of additional bases (bold text, Figure 4.17). These results showed that the plasmids pConstruct1 and pConstruct2 contained all of the sequence elements required for correct splicing and indicate that they should be spliced normally in transient transfection assays.



Key:
5' splice donor site
5' splice acceptor site
Branchpoint sequence
Ovine PrP ORF sequence
Additional bases to maintain reading frame (bold text)
3XFLAG™ epitope amino acids

Figure 4.17 *Line-up of pConstruct 1 plasmid DNA & Suffolk PrP gene DNA sequences (GenBank accession number = U67922)*

Figure shows the sequence line-ups of the pConstruct 1 DNA with: 1. Suffolk sheep (Genbank = U67922) promoter/exon I sequence. 2. pT7/T3α-18MS promoter sequence used in the cloning of the 0.5 kb Cheviot promoter fragment. 3. Suffolk sheep (U67922) exon III sequence.

4.2.2 Transient transfection of ovine PrP mini-gene constructs (pConstruct1-pConstruct5)

A wide variety of techniques are available to achieve the delivery of DNA molecules into cultured cells. However, no single method will work for all cell types and the conditions used must be optimised for each individual cell culture. There are two basic methods for introducing nucleic acids into cultured cells; biochemical and physical methods. Biochemical methods include DEAE-dextran, calcium phosphate, and liposome mediated transfection methods, whilst physical transfection methods include direct microinjection, biolistic particle delivery, and electroporation.

Previous studies by Marshall (2000) analysed the ability of a selection of these techniques to transfect the ovine cell cultures (sA80BR, IS120Liv and IS120Cer) with a plasmid consisting of the ovine PrP 0.5 kb promoter linked to a CAT reporter gene. Marshall (2000) showed that the Superfect transfection reagent (Qiagen, UK) was the most efficient method of transfecting the ovine cell cultures, showing a 40-80 % transfection efficiency with the sA80BR cell culture and with no observed cell death. It was decided to use Superfect transfection reagent for the transient transfection of the ovine PrP mini-gene DNA into the ovine cell cultures. However, it was first necessary to optimise the transfection conditions for each of the ovine cell cultures (sA80BR, pA80BR, IS120Liv, IS120Med and IS120Cer, Sections 2.5.1-2.5.3). The two most important aspects of the Superfect transfection process to be optimised are the amount of DNA added and the charge ratio of Superfect to DNA, and these factors were optimised for all of the ovine cell cultures and for the murine N2a cells.

4.2.3 Optimisation of transient transfection conditions for ovine & murine cell cultures

The reporter vector pSV-β-galactosidase (Promega, UK) was used to optimise Superfect transfection conditions in the cell cultures by analysing reporter activity in transfected cell lysates using the β-Galactosidase enzyme assay system (Promega, UK). The assay involves incubating the extract with the β-galactosidase enzyme substrate ONPG (*o*-nitrophenyl-β-D-galactopyranoside), β-galactosidase

then hydrolyses the colourless substrate to *o*-nitrophenyl, which is yellow in colour. The colour reaction is then assayed by measuring the absorbance at 420 nm (Section 2.6.3). Optimisation was carried out as previously described (Section 2.6.7), using a range of DNA concentrations and differing volumes of Superfect reagent. The optimum conditions for each of the cell cultures are detailed below (Table 4.1).

The vector pSV-β-galactosidase was then used to control for transfection efficiency, and the cell cultures were co-transfected with the ovine PrP mini-gene DNA and the pSV-β-galactosidase DNA at a ratio of 2:1. The assumption was made that the reporter vector and the ovine PrP mini-gene construct DNA would be transfected with equal efficiency in the cell cultures, but that the different cell populations would be transfected with differing efficiencies. Therefore, measuring the amount of β-galactosidase activity in the transfected cell extracts allowed the level of transfection efficiency to be determined for an individual cell lysate. The volume of each cell lysate used for protein analysis was then adjusted according to the efficiency of the transfection reaction. This was achieved by giving a score of 100 to the extract with the lowest absorbance value at 420 nm (therefore considered as having the lowest transfection efficiency) and all of this extract was added into the immunoprecipitation reaction. The volume of the other transfected cell extracts added into the immunoprecipitation reactions was then corrected accordingly, by dividing their absorbance values by the absorbance of the lowest efficiency extract. This value was then divided by the total volume of the extract (~ 250 µl following the use of 250 µl for the β-Galactosidase assay) to give a volume in microlitres of the extract to be added for immunoprecipitation.

Cell culture	Optimum DNA concentration (μg)	Optimum volume of Superfect (μl)	Optimum Superfect: charge ratio
sA80BR	4	20	5:1
pA80BR	4	20	5:1
IS120Liv	4	8	2:1
IS120Cer	4	20	5:1
IS120Med	4	40	10:1
Murine N2a	4	20	5:1

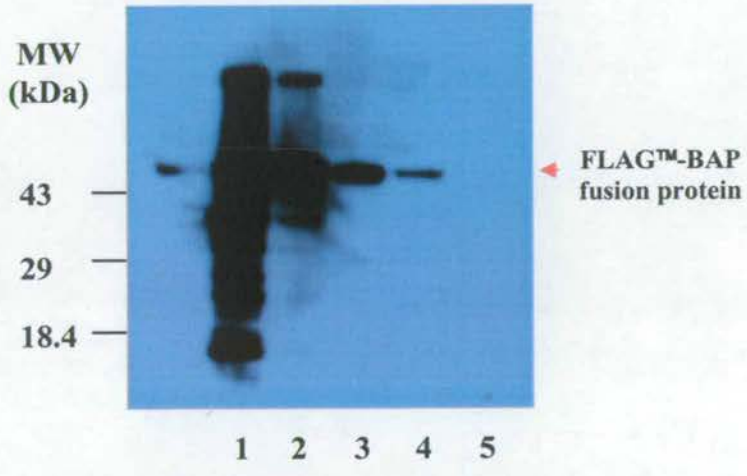
Table 4.1 *Optimum conditions for Superfect transfection of ovine and murine cell cultures*

Details the optimum transfection conditions with Superfect for each of the ovine and murine cell lines as determined by transient transfection with the pSV-β-galactosidase plasmid. Transfection efficiency was then calculated by measuring the amount of β-galactosidase enzyme activity in the cell lysates of the transfected cell lines for each of the DNA concentrations and volumes of Superfect as detailed in the table.

4.2.4 Optimisation of anti-FLAG™ antibody conditions for detection of 3XFLAG™ tagged PrP^c by Western blot

Attempts to detect 3XFLAG™ tagged PrP^c with an anti-FLAG™ monoclonal antibody (M2, Sigma-Aldrich, UK) following transfections with the ovine PrP mini-gene constructs in ovine sA80BR and IS120Cer, and murine N2a cell cultures failed (data not shown). These initial experiments were repeated again using the manufacturers recommended conditions for Western blotting with the M2 antibody, however, no 3XFLAG™ PrP^c was detected in the transfected cell lysates, and the background observed on the Western blot with the M2 antibody was very high (data not shown). It was decided to optimise the blotting conditions for the M2 antibody for the detection of the 3XFLAG™ epitope. A control FLAG™ protein (amino-terminal FLAG™-bacterial alkaline phosphatase (BAP) fusion protein (FLAG™-BAP), molecular weight 49 kDa, Sigma-Aldrich, UK) was utilised to optimise the Western blotting conditions for the M2 antibody. The FLAG™-BAP protein would also serve as a positive control protein on Western blots, as such it was important to

determine the optimum amount of FLAG™-BAP protein to load as a positive control. The dilution of the M2 antibody recommended by Sigma-Aldrich is 1:500, however, the use of the antibody at this dilution resulted in very high levels of background (data not shown). It was therefore decided to attempt to detect the FLAG™-BAP protein with the M2 antibody on Western blots (Section 2.8) at dilutions of 1:2500 and 1:5000 in order to reduce the background reaction (Figure 4.18, only 1:5000 blot shown). The secondary antibody used throughout this chapter was a peroxidase conjugated affinipure rabbit anti-mouse IgG (Stratech, UK) and this was used with both the 6H4 (anti-PrP) and the M2 (anti-FLAG™) monoclonal antibodies.

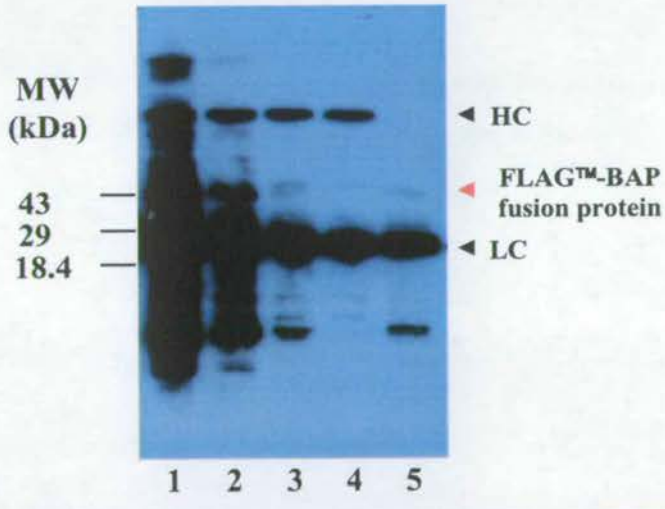


KEY: Lane 1, FLAG-BAP control protein (1 µg); Lane 2, FLAG™-BAP (100 ng); Lane 3, FLAG™-BAP (10 ng); Lane 4, FLAG™-BAP (1 ng); Lane 5, FLAG™-BAP (100 pg). Primary antibody = M2 (anti-FLAG™) at 1:5000 dilution. Secondary antibody at 1:25,000.

Figure 4.18 *FLAG™-BAP control protein detected by Western blot with anti-FLAG™ M2 antibody*

Western blot shows a dilution series of the FLAG-BAP control protein (1 µg – 100 pg). Red arrowhead indicates the location of the FLAG-BAP control protein (Molecular weight = 49 KDa).

The use of the M2 antibody at a dilution of 1:5000 resulted in the detection of the FLAG™-BAP protein (Figure 4.18). The FLAG™-BAP protein was observed over a range of concentrations down to 1 ng, but not at the 100 pg level (Figure 4.18). The use of the M2 antibody at a dilution of 1:2500 resulted in similar levels of FLAG™-BAP protein detection, however, the background reaction was elevated (data not shown). The optimum concentration of the FLAG™-BAP protein would appear to be between 10 ng and 1 ng, as at these levels the protein is clearly detectable and the background is relatively low in comparison (Figure 4.18). The ability of the M2 antibody to detect the FLAG™-BAP protein was further analysed by immunoprecipitating the FLAG™-BAP protein with the M2 antibody (Section 2.7.3). The samples were analysed on Western blot (Section 2.8) for the presence of the immunoprecipitated FLAG™-BAP protein using M2 as the detecting antibody (Figure 4.19).

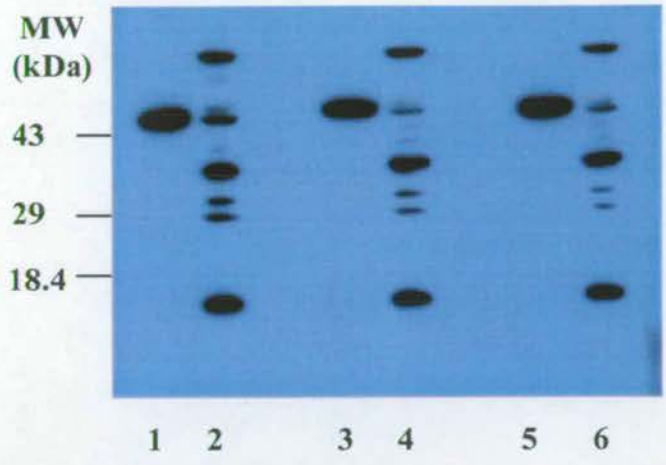


KEY: Lane 1, FLAG™-BAP control protein (100 ng) + M2 antibody as precipitating antibody; Lane 2, FLAG™-BAP (10 ng) + M2 antibody; Lane 3, FLAG™-BAP (1 ng) + M2 antibody; Lane 4, M2 antibody only; Lane 5, FLAG™-BAP (1 ng) only. Primary antibody = M2 (anti-FLAG™) at 1:5000 dilution. Secondary antibody at 1:25,000. LC = precipitating antibody light chain, HC = precipitating antibody heavy chain.

Figure 4.19 *FLAG™-BAP control protein immunoprecipitated with anti-FLAG™ M2 antibody*

Western blot shows a dilution series of the FLAG-BAP control protein immunoprecipitated with the anti-FLAG M2 monoclonal antibody and detected with the same antibody. Red arrow indicates the location of the immunoprecipitated FLAG-BAP control protein (Molecular weight 49 KDa).

As can be seen from Figure 4.19 it appeared that the anti-FLAG™ monoclonal antibody M2 was successful at immunoprecipitating the FLAG™-BAP protein, and again the optimum concentration of control was between 10 ng and 1 ng. The levels of background on this blot were notably high and two of these bands (25 and 50 kDa) are likely to be the result of cross reactivity between the secondary antibody and the light (LC) and heavy chains (HC) of the precipitating M2 antibody (Figure 4.19). It was therefore decided to try and further dilute the M2 antibody for Western blotting and to include on these Westerns, molecular weight markers as control proteins, in order to assess the M2 antibodies cross reactivity with non-specific proteins (data not shown). The results showed that M2 reacted with proteins in the molecular weight marker mixture at dilutions of between 1:500- 1:2500, whilst the antibody still reacted with the FLAG™-BAP protein (1 ng) at these concentrations (data not shown). When M2 was diluted to between 1:5000 and 1:7500 the cross reactivity with the molecular weight markers was prevented, and the antibody was still able to detect the FLAG™-BAP protein (1 ng). These results indicated that M2 would still function if diluted out to 1:7500 but that the background reaction may be reduced. This was tested further, by analysing the ability of M2 to cross react with elements in raw M-per produced (Section 2.5.9.3) N2a cell lysates (Figure 4.20). M2 was diluted out from 1:5000 to 1:10,000 and a 15 µl aliquot of a ~ 500 µl M-per produced N2a cell lysate was loaded adjacent to 1 ng of FLAG™-BAP protein at each antibody dilution (Figure 4.20). Even at a dilution of 1:10,000 M2 was still able to detect 1 ng of FLAG™-BAP protein, however, even at this dilution the background observed with the N2a cell lysate was still very high, raising concerns about the quality of the M2 antibody and its ability to function in this system without producing high levels of background.



KEY: Lanes 1, 3 & 5, FLAG™-BAP control protein (1 ng); Lanes 2, 4 & 6, 15 µl of a ~ 500 µl M-per produced N2a cell lysate. Primary antibody = M2 (anti-FLAG™), Lanes 1-2, 1:5000 dilution; Lanes 3-4, 1:7500 dilution; Lanes 5-6, 1:10,000 dilution. Secondary antibody, 1:25,000.

Figure 4.20 *FLAG™-BAP control protein and N2a cell lysates*

Western blot shows 1 ng of FLAG-BAP control protein alongside 15 µl of a ~ 500 µl M-per produced N2a cell lysate detected using the anti-FLAG M2 antibody. As can be seen 1 ng of the FLAG-BAP control protein produces a strong signal, however the N2a cell lysate also produces a high background signal with the M2 antibody, indicating a lack of specificity.

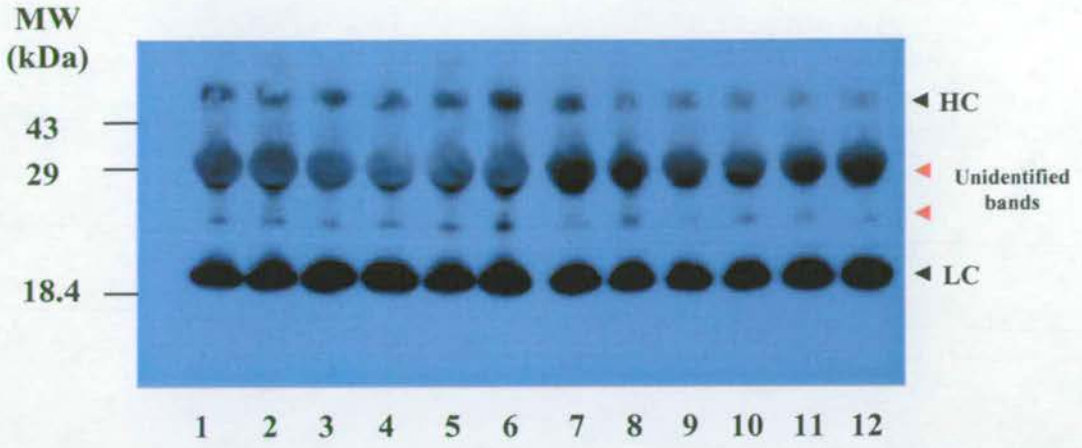
An anti-FLAG™ polyclonal antibody (Sigma-Aldrich, UK) was tried as an immunoprecipitating antibody as it was hoped that this would provide more options for immunoprecipitating the 3XFLAG™ tagged PrP^c expressed from the ovine PrP mini-gene constructs. In addition, it would provide the option of using of an anti-PrP monoclonal antibody like 6H4 as the detecting antibody, which may get around the problem of high background observed with M2 when used as a detecting antibody (Figure 4.20). Attempts to immunoprecipitate (Section 2.7.3 & 2.8) the FLAG™-BAP protein using the anti-FLAG™ polyclonal antibody were inconclusive (data not shown). The antibody did not successfully immunoprecipitate the FLAG™-BAP protein and it appeared to react strongly with the secondary antibody, resulting in strong light (LC) and heavy chain (HC) bands on the Western blots. In addition, high levels of background were observed with the M2 antibody on samples

immunoprecipitated with the anti-FLAG™ polyclonal antibody (data not shown). Finally, it was decided to optimise the conditions for the M2 antibody using the FLAG™-BAP protein on dot blots (Section 2.8.4, data not shown). The dot blots confirmed that the optimum dilution of the M2 primary antibody for detecting down to 1 ng of FLAG™-BAP protein was 1:5000. Dot blots (Section 2.8.4) were also used to optimise the dilution of the secondary antibody whilst using the primary antibody at the optimum dilution of 1:5000, the results showed that the optimum dilution of the secondary antibody was 1:20,000 (data not shown). It was decided to attempt to detect 3XFLAG™ tagged PrP^c in cell cultures transiently transfected with the ovine PrP mini-gene constructs using the optimum antibody dilutions determined from the dot blots.

4.2.5 *In vitro* analysis of recombinant 3XFLAG™ tagged PrP^c in ovine and murine cell cultures by Western blotting

The expression of 3XFLAG™ tagged recombinant ovine PrP^c was analysed from ovine and murine N2a cell cultures transfected (Section 2.6) with the ovine PrP mini-gene constructs. Cultured IS120Liv and murine N2a cells were transiently transfected with the plasmids pConstruct1-pConstruct5, using 20 µl (N2a cells) or 8 µl (IS120Liv cells) of Superfect reagent (Section 2.6.5). In addition, a total of 4 µg of DNA, at a ratio of 2:1 of ovine PrP mini-gene construct DNA: reporter vector DNA, was added (i.e. 2.6 µg of mini-gene construct DNA and 1.4 µg of β-galactosidase reporter DNA). Following incubations of 3, 6 or 24 hours the transfected cells were lysed with Mem-per reagent (Section 2.5.9.4) and transfection efficiency, as determined by β-galactosidase activity (Section 2.6.3), was measured in the cell extracts. The amount of each individual cell lysate added into the immunoprecipitation reaction was determined, as described in Section 4.2.3, and the cell extracts were analysed for recombinant PrP^c expression. Recombinant ovine PrP^c was isolated by immunoprecipitation using the PrP specific polyclonal antibody 1A8 (Section 2.7.3). Immunoprecipitated proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and recombinant PrP^c was detected on Western blot (Section 2.8) with the anti-FLAG™ M2 monoclonal antibody (using the

optimum primary and secondary antibody dilutions as determined by dot blots in Section 4.2.4). Figure 4.21 shows N2a cells transfected with the plasmids pConstruct1-pConstruct5, which were analysed for the expression of 3XFLAG™ tagged PrP^c expression following 3 hour, or 6 hour post-transfection incubation periods. The transfections did not appear to have been successful, and no 3XFLAG™-tagged PrP^c was detected (Figure 4.21). Two antibody bands were observed, which represent the light (LC) and heavy chains (HC) of the precipitating 1A8 antibody (Figure 4.21). Two bands (marked with red arrowheads) can be seen located between the two antibody bands, however, these bands are also present in the cell lysates that were transfected with, either β-galactosidase DNA only, or with no DNA (Figure 4.21). These bands may represent a cross-reaction of the anti-FLAG™ M2 antibody with unknown proteins present in the immunoprecipitated N2a cell (Figure 4.21).



KEY: N2a cell extracts prepared with Mem-per and transfected with the stated plasmid. Lanes 1+ 7, pConstruct5 DNA; Lanes 2+ 8, pConstruct2 DNA; Lanes 3 + 9, pConstruct4 DNA; Lanes 4 + 10, pConstruct1 DNA; Lanes 5 + 11, β-galactosidase DNA only; Lanes 6 + 12, no DNA; Lanes 1-6 were analysed 3 hours post transfection; Lanes 7-12 were analysed 6 hours post transfection. Primary antibody = M2 (anti-FLAG™), 1:5000 dilution, Secondary antibody 1:20,000 dilution. LC = precipitating antibody light chain, HC = precipitating antibody heavy chain

Figure 4.21 *Transfected N2a cell lysates immunoprecipitated with 1A8, analysed for 3XFLAG™ tagged PrP^c, 3 & 6 hours post transfection*

Western blot shows Mem-per extracts from N2a cells transfected with the ovine PrP mini-gene construct DNA, cell extracts were produced 3 & 6 hours post-transfection. As can be seen two unidentified bands (Red arrowheads) were observed between the bands for the light (LC) and heavy chain (HC) bands of the precipitating antibody (1A8), however these bands are also present in the control samples, indicating that they do not correspond to recombinant PrP.

Figure 4.22 shows N2a cells transfected with the plasmids pConstruct1-pConstruct5 (Section 2.6) which were analysed for the expression of 3XFLAG™ tagged PrP^c expression following a 24 hour post-transfection incubation period (Section 2.7.3 & 2.8). Again the transfections do not appear to have been successful, and no 3XFLAG™-tagged PrP^c was detected (Figure 4.22). The two antibody bands representing the light (LC) and heavy chains (HC) of the precipitating 1A8 antibody were again observed (Figure 4.22). The same bands (marked with red arrowheads), as observed in Figure 4.21, are present at a molecular weight indicative of PrP^c, however this band is also present in the non-transfected control samples. Again, as in

Figure 4.21, it is likely that these bands represent a cross-reaction of the anti-FLAG™ M2 antibody with unknown proteins present in the cell extracts.

KEY: N2a cell extracts prepared with Mem-per and transfected with the stated plasmid. Lanes 1-2, pConstruct5 DNA; Lanes 3-4, pConstruct2 DNA; Lanes 5-6, pConstruct4 DNA; Lane 7, FLAG™-BAP control protein (10 ng); Lane 8, Purified PrP^{sc} control sample (20 mg); Lanes 9-10, pConstruct1 DNA; Lanes 11-12, pConstruct3 DNA; Lanes 13-14, non-transfected N2a cells. Primary antibody = M2 (anti-FLAG™), 1:5000 dilution, Secondary antibody 1:20,000 dilution. LC = precipitating antibody light chain, HC = precipitating antibody heavy chain

Figure 4.22 *Transfected N2a cell lysates immunoprecipitated with 1A8 and analysed for 3XFLAG™ tagged PrP^c, 24 hours post transfection*

Western blot shows Mem-per extracts from N2a cells transfected with the ovine PrP mini-gene construct DNA, cell extracts were produced 24 hours post-transfection. As can be seen two unidentified bands (Red arrowheads) were observed between the bands for the light (LC) and heavy chain (HC) bands of the precipitating antibody (1A8), however these bands are also present in the control samples, indicating that they do not correspond to recombinant PrP.

No 3XFLAG™ tagged PrP^c, was detected in any of the transfected cell cultures. Negative results were observed with all of the transfections using the PrP specific polyclonal antibody 1A8 as an immunoprecipitating antibody and the anti-FLAG™ M2 antibody to detect 3XFLAG™ tagged PrP^c expression from the transfected cell lysates. A variety of cultured cells were analysed, including, murine N2a, ovine sA80BR, IS120Cer and IS120Liv, and unfortunately none of these

transfected cell cultures have indicated any expression of recombinant PrP^c from the ovine PrP mini-gene constructs (only N2a data shown). As it was not possible to detect 3XFLAG™ tagged PrP^c from the transiently transfected ovine and murine cell cultures, it was decided to check for the presence of PrP mini-gene construct mRNA in transiently transfected cell cultures using reverse transcriptase-PCR (RT-PCR).

4.2.6 *In vitro* analysis of ovine PrP mini-gene construct mRNA by RT-PCR

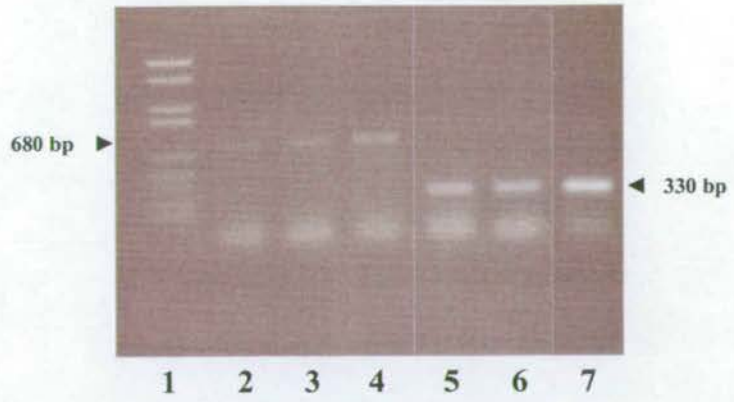
The expression of ovine PrP mini-gene construct mRNA was analysed by RT-PCR (Section 2.2.16) in IS120Liv cells transiently transfected with the plasmids pConstruct1 and pConstruct2 (Section 2.6). The ovine PrP specific primers used for the reverse transcription (RT) reaction were either AG23 or 643 (Table 2.1). The primers used in the PCR reaction were AG23, 580SH, 13741 or Flag Reverse (Table 2.1). The two, 3XFLAG™ specific primers are specific for the ovine PrP mini-gene construct mRNA reverse transcriptase products, whilst the PrP specific primers (AG23, 580SH & 13741) will react with both endogenous PrP mRNA and ovine PrP mini-gene construct mRNA RT products. The expected size of the RT-PCR products from the mini-gene RNA with the primers AG23 and 13741 (if the RNAs are correctly spliced) was approximately 230 bases (Table 4.2). If splicing of the construct RNAs failed then a product of approximately 1000 bases would be expected as the 3' sequence of intron II would not be removed (encoded by 713 bases plus 27 bases of the multi-cloning site of the plasmid pNPU-110, between the 0.5 kb promoter fragment and the ovine PrP gene exon III fragment) (Table 4.2). The expected size of the RT-PCR products with the primers AG23 and 580SH if the construct RNAs were correctly spliced was 580 bases and 1300 bases if not correctly spliced (Table 4.2). Finally the fragments expected with AG23 and Flag Reverse would be 215 bases if the construct RNAs were correctly spliced and 1000 bases if incorrectly spliced (Table 4.2). Endogenous PrP mRNA from the cultured cells would produce a RT-PCR product of approximately 330 bases with the primers AG23 & 13741, and 680 bases with the primers AG23 & 580SH (identical to that seen with the correctly spliced construct RNA plus 100 bases of ovine PrP gene exon II DNA).

Primer combination	Size (bp) if correctly spliced	Size (bp) if incorrectly spliced
AG23 + 13741	230 mini-gene mRNA	1000
	330 endogenous PrP mRNA	
AG23 + 580SH	580 mini-gene mRNA	1300
	680 endogenous PrP mRNA	
AG23 + Flag Reverse	215	1000

Table 4.2 *Expected sizes of RT-PCR products from ovine PrP mini-gene mRNA*

Details the expected product size following RT-PCR with the stated primer combinations. Expected sizes are detailed for the correctly spliced and incorrectly spliced ovine PrP mini-gene mRNA and for the endogenous PrP mRNA in bp.

Prior to transient transfections with the ovine PrP mini-gene construct DNA, RNA was isolated from cultured IS120Liv cells (Section 2.5.11) and the presence of endogenous PrP mRNA was confirmed by RT-PCR (Figure 4.23). These results were compared to PCR products from ovine kidney cDNA (Wilfred Goldmann, NPU) using the PrP specific primer AG23 for the RT reaction and AG23 & 13741, and AG23 & 580SH for the PCR reactions (Figure 4.23). RT-PCR products of the expected sizes from endogenous PrP mRNA (approximately 330 bases for AG23 & 13741 and approximately 680 bases for AG23 & 580SH) were detected confirming that endogenous PrP mRNA was detectable by this method in the IS120Liv cells (Figure 4.23).

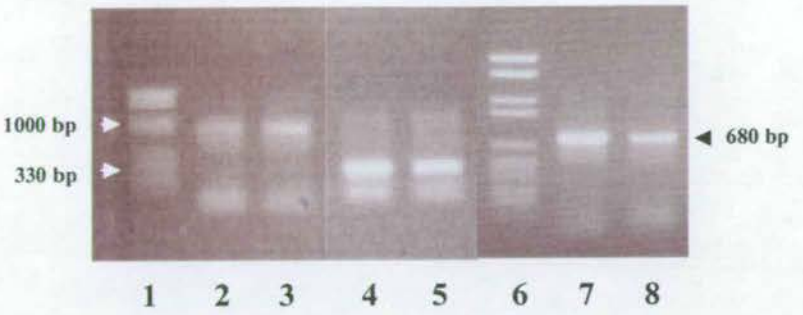


KEY: Ovine IS120Liv cell RNA samples analysed by RT-PCR. Lane 1, Molecular weight marker VI (Roche, UK); Lanes 2-3, IS120Liv RNA using AG23 & 580SH primers; Lane 4, Ovine kidney cDNA sample using AG23 & 580SH primers; Lanes 5-6, IS120Liv RNA using AG23 & 13741 primers; Lane 7, Ovine kidney cDNA using AG23 & 13741 primers.

Figure 4.23 *RT-PCR analysis of IS120Liv cell RNA for endogenous ovine PrP mRNA expression*

Agarose gel shows untransfected ovine IS120Liv (liver) cell total RNA analysed for the presence of endogenous PrP mRNA expression, expected sizes for RT-PCR fragment are: 680 bp using the AG23 & 580SH primers or 330 bp using the AG23 & 13741 primers.

Cultured IS120Liv cells were transiently transfected with the plasmids pConstruct1 and pConstruct2, using 8 µl of Superfect reagent and a total of 4 µg of mini-gene construct DNA per transfection (Section 2.6). Following a 24-hour incubation, RNA was isolated from the cells using RNAzol reagent (Section 2.5.11). The RT reaction was performed on the isolated RNA samples, using the ovine PrP ORF specific primer 643 (Section 2.2.16). The PCR reactions (Figure 4.24) were carried out using the following primer combinations AG23 & 580SH, AG23 & 13741, and AG23 & Flag Reverse and the expected fragment sizes are detailed in Table 4.2.



KEY: RNA isolated from ovine IS120Liv cells transiently transfected with plasmids pConstruct1-pConstruct5, analysed by RT-PCR. Lanes 1 + 6, Molecular weight marker VI (Roche, UK); Lane 2, pConstruct2 using AG23 & Flag Reverse primers; Lane 3, pConstruct1 using AG23 & Flag Reverse primers; Lane 4, pConstruct2 using AG23 & 13741 primers; Lane 5, pConstruct1 using AG23 & 13741 primers; Lane 7, pConstruct2 using AG23 & 580SH primers; Lane 8, pConstruct1 using AG23 & 580SH primers.

Figure 4.24 *RT-PCR analysis of transiently transfected IS120Liv cell RNA for ovine PrP mini-gene mRNA expression*

Agarose gel shows total RNA from ovine IS120Liv (liver) cells transfected with ovine PrP mini-gene construct DNA analysed for the presence of recombinant PrP mRNA expression. Expected sizes for RT-PCR fragment are: 230 bp if spliced correctly, 330 bp endogenous PrP mRNA or 1000 bp non-spliced using the AG23 & 13741 primers. 580 bp if correctly spliced, 680 bp endogenous PrP mRNA or 1300 bp if non-spliced. 215 bp if correctly spliced or 1000 bp if non-spliced using the AG23 & Flag Reverse primers. As can be seen only bands corresponding to non-spliced and endogenous PrP mRNA were observed and at no time were bands corresponding to the correctly spliced mRNA seen.

As can be seen in Figure 4.24, RT-PCR failed to reveal bands of the expected size for correctly spliced ovine PrP mini-gene RNA, instead bands of approximately 1000 bases with AG23 and Flag Reverse primers were observed, indicating one of two possibilities: i) that only non-spliced construct RNA was present or ii) that due to the failure to treat the RNA samples with DNase the 1000 base band corresponds to amplified ovine PrP mini-gene construct DNA. Bands of approximately 1000 bases (faint) and 330 bases were observed with AG23 and 13741 primers, indicating that endogenous PrP mRNA (330 bases) and either non-spliced construct mRNA or contaminating ovine PrP mini-gene construct DNA (1000 bases) bands were present.

Finally only endogenous PrP mRNA bands (680 bases) were observed with the 580SH and AG23 primers, indicating that neither spliced, nor non-spliced construct mRNA or indeed contaminating ovine PrP mini-gene construct DNA was detectable with this primer combination (Figure 4.24).

4.2.7 Sequencing of ovine PrP mini-gene construct mRNA RT-PCR products

In order to confirm that the ovine PrP mini-gene construct RNA had not been correctly spliced, the pConstruct1 and pConstruct2 RT-PCR products (Section 4.2.6) were sequenced on an ABI 377 automatic sequencer (Sections 2.3.5-2.3.6). Sequencing was carried out with the primers B007 and AG28 (Table 2.2). The interpretation of the results is affected by the fact that the tested RNA samples were not treated with DNase, and thus any RT-PCR products could have originated from contaminating ovine PrP mini-gene construct DNA. One interpretation is therefore that the construct RNAs had not been correctly spliced as the intron II sequence was still intact 5' to the ovine PrP mini-gene exon III DNA and the splice donor, splice acceptor sites and the branchpoint sequence were also present (data not shown). However, it could also be that what was amplified was in fact from the construct DNA and would not be expected to be spliced as a result.

4.3 Discussion

Previous work identified a potential role for the 3'UTR in the regulation of PrP gene expression (Goldmann *et al.*, 1999, Marshall, 2000). It is hypothesised that the differential use of alternative polyadenylation sites incorporates sequence elements into the 3'UTR that exert differing functions. In order to investigate the regulated control of alternative polyadenylation and the function of the 3'UTR sequence elements in PrP gene expression, a series of ovine PrP mini-gene constructs were produced (pConstruct1-pConstruct5). The expression of these constructs was placed under the control of a 0.5 kb ovine PrP gene promoter fragment, which was known to be functional (Marshall, 2000, O'Neill *et al.*, 2003). In addition, the constructs were designed to express recombinant ovine PrP^c tagged with the

3XFLAG™ epitope within its N-terminal region to allow the detection of recombinant PrP^c against a background of endogenous PrP^c.

Previous work by Telling *et al.* (1997) had shown that the incorporation of a single FLAG™ epitope at the N-terminus of the mature murine PrP protein (in a position similar to that utilised in the ovine PrP gene in this study) had no effect on its normal cellular processing. It was shown that it was possible to immuno-purify FLAG™ tagged murine PrP^c from the brains of transgenic mice using anti-FLAG™ monoclonal antibodies (Telling *et al.*, 1997). Any tagging epitope used had to be capable of being inserted and detected at a position that would not interfere with the normal processing of PrP^c. The insertion of the 3XFLAG™ epitope C-terminal of the signal peptide sequence ensured that it would not be cleaved following the post-translational modification of PrP. Another reason for inserting the 3XFLAG™ epitope in the N-terminal region of PrP, was that this section of the PrP^c protein is believed to be relatively unstructured (Mehlhorn *et al.*, 1996, Pergami *et al.*, 1996). It was hoped that this would reduce the possibility of the 3XFLAG™ epitope becoming buried within the fully folded PrP^c protein. The cleavage of the N-terminal signal sequence in mature PrP^c ruled out the use of other tagging epitopes (such as 6 x His), which could only be inserted at the N- or C-terminus of the recombinant protein. The reason for this is that these tagging epitopes would either have been removed during post-translational modification (N-terminal attachment), or may have interfered with the membrane attachment of the mature protein via its GPI-anchor (C-terminal attachment). The 3X FLAG™ epitope had been shown to be a more sensitive method of detection of recombinant protein than other tagging epitopes such as 6 x His and FLAG™ (Hernan, Heuermann & Brizzard, 2000, Zhang, Hernan & Brizzard, 2001).

Transient transfection of the ovine and murine cell cultures with the ovine PrP mini-gene constructs was used as a model to examine the activity of the 3'UTR in differing cell types. The expression of these constructs in a range of ovine cell types may offer some clues as to the role of alternative polyadenylation in the tissue-specific expression of the PrP gene, and could provide further insights into the mechanisms of scrapie disease pathogenesis. Methods for the immunoprecipitation of endogenous PrP^c from ovine & murine cell cultures, and murine tissue were developed during this study (Chapter 3). These techniques were applied to the

isolation of the recombinant 3XFLAG™-tagged PrP^c from the transiently transfected cells, using PrP- and FLAG™ specific antibodies. However, the use of the 3XFLAG™ tag proved to be very problematic and major difficulties were encountered whilst attempting to detect a purified control FLAG™-BAP protein using the M2 antibody (Figures 4.18 & 4.19). The level of background observed with this antibody was very concerning, and even at very low concentrations the M2 antibody still reacted non-specifically with raw N2a cell lysates (Figure 4.20). It is not clear why this cross reactivity occurred but it could be due to the presence of endogenous FLAG™ like epitopes in the cell cultures analysed, thus providing serious problems for the detection of FLAG™ tagged proteins in this system. Following these problems, literature searches revealed that other researchers had encountered problems with the anti-FLAG™ M2 antibody. For example, Schafer & Braun (1995) showed that the Mg^{2+} dependent protein phosphatase (MPP) reacted strongly on Western blot with the anti-FLAG™ M2 antibody. Furthermore, sequence analysis of MPP revealed a sequence motif containing five out of eight amino acid residues, which were identical to the FLAG™ epitope (Schafer & Braun, 1995). These results indicated that the FLAG™ epitope may not be mono-specific, as was previously thought and that naturally occurring homologues of this sequence may exist.

No 3XFLAG™-tagged PrP^c expression was detected in any of the transiently transfected cell cultures (Figures 4.21 & 4.22), including the ovine IS120Liv cells. The ovine IS120Liv cell culture was a model system for the expression of recombinant PrP^c as it had shown no detectable PrP^c, thus providing an effective PrP null background (Chapter 3). However, it could be argued that the liver cells, in which there was no endogenous PrP^c expression, did not support the expression of the transfected ovine PrP mini-genes. The results were unexpected as a series of ovine PrP mini-gene constructs similar to those produced in this study, but without the 3XFLAG™ tag, had previously been shown to be correctly transcribed and spliced during a previous PhD project (Marshall, 2000). In addition, the transfection reagent selected (Superfect) had previously been shown to be very effective at transfecting the ovine cell cultures (Marshall, 2000) and the conditions for its use had been optimised for each individual cell culture during the present study. Sequencing

of the ovine PrP mini-gene constructs (plasmids pConstruct1 & pConstruct2) confirmed the sequence integrity of these constructs and showed the presence of all of the required sequence elements identified to date. These included, 5' & 3' splice sites, a branch-point sequence, and the plasmids also contained an ovine PrP gene intron region of approximately 700 bases (Figure 4.17). RT-PCR analysis of transiently transfected ovine cell culture RNA failed to detect any correctly spliced construct RNA (Figures 4.23 & 4.24) but the experiment was compromised by the likely presence in the reaction mixture of contaminating construct DNA due to the failure to treat the RNA samples with DNase. In addition there was no control sample included without the use of the reverse transcriptase reaction (a minus RT control).

Due to the absence of important controls it is still unclear if the ovine PrP mini-gene construct RNAs were being correctly spliced or not, however incorrect splicing could have been due to the design of the constructs themselves, as they could be lacking sequence elements other than those identified in this study. For example, the constructs are missing most of the ovine PrP gene intron I, all of exon II and some of intron II, and the presence of sequence elements required for the correct processing of PrP mRNA in these sequences cannot be ruled out. Inoue *et al.* (1997) identified elements within the bovine PrP gene intron I that were required, in conjunction with the bovine PrP gene promoter, for the efficient expression of the bovine PrP gene. In addition, Fischer *et al.* (1996) showed that in mice the PrP gene intron II was vital for the expression of PrP^c in cerebella Purkinje cells, and mice that lacked the intron II region failed to show detectable levels of PrP^c expression. The failure of the ovine PrP mini-gene constructs, generated during this study, to successfully express recombinant PrP^c could be due to an inherent problem with the constructs themselves that has not been identified to date. The constructs were produced in a way that ensured that the reading frames of the ovine PrP gene ORF, and the 3XFLAG™ epitope were maintained. However, in order to maintain reading frame of the 3XFLAG™ epitope sequence it was necessary to repeat three codons ($Tyr_{41}Pro_{42}Gly_{43}$) of the ovine PrP gene ORF (Figure 4.10). Although reading frame was maintained it is unlikely that the introduction of these extra bases into the PrP

ORF could have had a detrimental affect on the expression of the ovine PrP mini-gene constructs.

Attempts were made during this study to produce a series of identical ovine PrP mini-gene constructs that contained a much more complete, 3.2 kb promoter fragment, including intron I and exon II (data not shown). However, this 3.2 kb promoter fragment proved to be very difficult to clone, the reasons for this are unclear and many different methods of introducing this fragment were attempted by myself and by other researchers at the NPU. As a result the production of this additional series of mini-gene constructs was halted, and only the constructs containing the 0.5 kb promoter were used in this study. The main reason for using a PrP mini-gene was that due to its small size, it could be easily transiently transfected into the cell cultures. Previous studies had shown that this was possible, and had identified the Superfect reagent as being the most efficient method of transfecting the ovine cell cultures (Marshall, 2000). This method allows cultured cells to be transfected in an efficient and reproducible manner with very little damage to the cells. However, by using a PrP mini-gene, the size constraints meant that certain regions of the gene, deemed to be unnecessary were omitted from the final constructs, i.e. most of intron I, exon II and most of intron II. The use of a full-length PrP gene would have been more desirable, as the omission of sequences within the introns may have led to the failure of the construct RNAs to be spliced correctly. However, a full-length PrP gene construct would have been some 30 kb in length and would have been much more difficult to clone, and to introduce into the ovine cell cultures.

4.3.1 Conclusions

Considering the problems that have been highlighted during this study, such as the lack of specificity observed with the FLAG™ antibodies, and a lack of obvious sequence defects in the constructs it was decided not to pursue the present constructs any further. It is possible however that DNase treatment would allow the RT-PCR reaction to work on the RNA rather than on any contaminating DNA and the constructs remain available for future studies. The possibility of producing a new series of constructs was ruled out due to the amount of cloning required and the time

that this would entail. In addition, it is possible that even if a working series of ovine PrP mini-gene constructs were produced, the lack of specificity of the anti-FLAG™ antibodies may have interfered with, or nullified the experimental model. The lack of specificity of the anti-FLAG™ antibodies could have been overcome by instead using ^{35}S-methionine labeling to detect recombinant PrP^c expression, however, the failure of the construct RNAs to be correctly spliced meant that this was not possible. As such it has not been possible to further investigate the role of the ovine PrP gene 3'UTR in the regulation of PrP gene expression during this study. Therefore, it was decided to concentrate on the remaining aspects of the thesis, which involved analysing the role of the ovine PrP gene promoter region in the regulation of PrP gene expression (Chapters 5 & 6).

Chapter 5: Characterisation of the ovine PrP gene promoter region

5.1 Introduction

The PrP gene promoter region regulates the expression of the PrP gene and this regulation is controlled by transcription factors. Gene expression is primarily regulated by specific sequence elements within the promoter region, which form binding motifs for transcription factors (Mitchell & Tjian, 1989). These factors either aid in the formation of the basal transcription complex, in conjunction with RNA polymerase II, or they act to regulate the rate of transcription of a particular gene through interactions with other transcriptional elements (Mitchell & Tjian, 1989, Roeder, 1996, Tjian, 1995, Zawel & Reinberg, 1995). Gene transcription is a highly complex process, and the number of factors involved allows for a large number of potential combinations of these factors to associate together on a given gene promoter (Hochheimer & Tjian, 2003, Tjian, 1995). However, only certain combinations will result in the transcription of a particular gene, and by limiting the availability of different factors in particular cell-types, an organism is able to regulate the tissue-specific expression of a given gene (Tjian, 1995). Gene transcription can also be regulated by controlling the cellular levels of transcription factors, and the expression of transcription factors can be influenced by a wide variety of extracellular stimuli (Kagoshima, Cosio & Adcock, 2003). These stimuli can include stress, ion concentrations and responses to viral infections (Morimoto, Sarge & Abravaya, 1992, Sassone-Corsi, 1995, Tamai, Monaco, Nantel *et al.*, 1997, Whitmarsh & Davis, 2000). In addition, the expression levels of some transcription factors has been shown to be regulated in a circadian manner (Cowell, 2002, Holzberg & Albrecht, 2003, Takahashi, 1993, Wuarin, Falvey, Lavery *et al.*, 1992).

To date, very few transcription factor binding motifs have been identified in the ovine PrP gene promoter. An upstream AP-2 motif, and a downstream AP-2 motif in Suffolk sheep have been noted (Figure 1.9) (O'Neill *et al.*, 2003). In addition, a polymorphic change of C to G within the downstream AP-2 motif exists in the Cheviot PrP promoter and appears to create an SP-1 binding motif (O'Neill *et*

al., 2003). The non-ruminant PrP gene promoters contain a single consensus binding motif for the transcription factor AP-1. In addition, all of the mammalian promoters, with the exception of the published Suffolk promoter, contain between two and three SP-1 binding motifs (Baybutt & Manson, 1997, Funke-Kaiser *et al.*, 2001, Inoue *et al.*, 1997, Mahal *et al.*, 2001, O'Neill *et al.*, 2003, Saeki *et al.*, 1996, Westaway *et al.*, 1994a). O'Neill *et al* (2003) performed a series of targeted deletions of a 0.5 kb Cheviot PrP gene promoter fragment linked to a CAT reporter gene. The deletion of a section of 99 bases from the 5' end of this promoter sequence resulted in an initial loss of promoter activity (O'Neill *et al.*, 2003). Furthermore, deletion of a region containing the upstream AP-2 binding motif resulted in a considerable reduction in promoter activity (O'Neill *et al.*, 2003). A number of other single nucleotide polymorphisms have been discovered in the ovine PrP gene promoter, including a change of T to C within conserved motif 1 and the significance of this motif is investigated in further detail in Chapter 6. A single base polymorphism of C to A is found in positive line (scrapie susceptible) sheep in the NPU Cheviot flock, although its functional significance is currently unknown. These polymorphisms may have an influence on the binding of transcription factors to the promoter and may therefore influence expression of the PrP gene and the biology of the disease.

During this study, the ovine PrP promoter was subjected to extensive sequence analysis in order to identify any known transcription factor binding motifs. This analysis was carried out using the GCG Wisconsin package, version 10.3 (Fuchs, Stoehr, Rice *et al.*, 1990), and with the TRANSFAC (www.transfac.gbf.de/TRANSFAC/, (Wingender, Chen, Hehl *et al.*, 2000, Wingender, Dietze, Karas *et al.*, 1996)) and the TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) databases. DNA sequence analysis was concentrated on the area of the ovine PrP promoter between 4000-7000 bp using the Suffolk PrP promoter numbering (GenBank accession number = U67922) which represents approximately 1 kb upstream and downstream of the core ovine PrP promoter identified by O'Neill *et al.* (2003).

The biochemical functionality (i.e. DNA-protein interaction) of the ovine PrP promoter binding motifs was assessed by the gel shift assay technique (Section 2.4.6). The gel shift assay involved the radiolabeling of a double stranded DNA

oligonucleotide containing the DNA sequence to be analysed. The gel shift assays could also have been performed using restriction fragments prepared from the 0.5 kb Cheviot PrP promoter, however, this was not possible due to a distinct lack of suitable restriction enzyme sites within this DNA sequence and, as such, a decision was made to use synthetic oligonucleotides. The double stranded, radiolabeled oligonucleotide was incubated in a binding assay with a nuclear extract; in this study the nuclear extracts were prepared from the ovine and murine cell cultures available at the NPU (Sections 2.5.1-2.5.3). The binding of nuclear factors to the labeled oligonucleotides was assessed by separating the reaction products by non-denaturing gel electrophoresis. Unbound radiolabeled oligonucleotides run freely to the bottom of the gel, whereas, oligonucleotides that have become complexed with protein factors have reduced mobility and are observed on the gel as a retarded complex, or gel shift. Specificity of binding was assessed by the use of unlabeled competitor oligonucleotides (same DNA sequence but not radiolabeled), and unlabeled non-competitor oligonucleotides (different DNA sequence but not radiolabeled). Upon the identification of binding, selected mutations were introduced into the DNA sequences in order to test the binding specificity and to help to elucidate which specific factor was involved. Sequence analysis and gel shift experiments carried out in this study would help to elucidate the role of the ovine PrP gene promoter in the transcriptional control of gene expression. In addition, the analysis of sequence polymorphisms within the ovine PrP promoter would provide further insights into the role of gene transcription in scrapie disease susceptibility.

5.2 Results

5.2.1 Sequence analysis of the ovine PrP promoter

Sequence analysis revealed the presence of numerous transcription factor binding motifs within the ovine PrP promoter sequence. All of the motifs identified were found within the DNA sequence lying approximately 500 bp upstream of the potential transcription start sites identified by Westaway *et al.* (1994b) and are detailed in Figure 5.1 (boxed motifs were identified during this study). The motifs found on the ovine PrP promoter included two heat shock elements (HSE-1 & HSE-

2), and binding motifs for a number of transcription factors including activator protein-1 (AP-1), early gene transcription factor-1 (EGR-1), and a GATA-1 factor binding motif (Amin, Ananthan & Voellmy, 1988, Angel & Karin, 1991, Sukhatme, Cao, Chang *et al.*, 1988, Tsai, Martin, Zon *et al.*, 1989, Yan, Small, Desplan *et al.*, 1996). The influence of the C to A polymorphism found in the positive line in the NPU Cheviot flock was analysed and discovered to lie within a binding motif for the signal transducers & activators of transcription (STAT) family of transcription factors (Figure 5.1).

The expression of the ovine PrP gene is controlled by a TATA-less promoter, therefore, the ovine PrP gene promoter sequence was searched for the presence of initiator (Inr) elements (Section 1.10.1), the consensus sequence for which is $PyPyA_{+1}NT/APyPy$ (where Py = pyrimidine) (Lo & Smale, 1996, Smale, 1997). Certain sequence changes in this consensus sequence can be tolerated, however, the core sequence for an Inr element is $PyA_{+1}NT/A$, where A_{+1} is the transcription start site. In addition, pyrimidines need to surround the sequence in at least two of the four positions detailed in the consensus (Smale, 1997). Westaway *et al.* (1994b) mapped approximate positions of the major start sites at positions 5666 & 5669 bp in the Suffolk PrP gene promoter (GenBank accession number = U67922). Interestingly, the start site of transcription in the bovine PrP gene has been mapped to a similar sequence to that seen in the ovine PrP gene ($TAGTT_{+1}GCCAGTCGCT$), and lies immediately downstream of the three bovine SP-1 motifs (Inoue *et al.*, 1997). Five sequences were found, which fitted the core sequence for an Inr element at the 3' end of the ovine PrP gene promoter region. Three of these sequences were located downstream of the 5' splice site and were therefore excluded as candidates, the other two are listed in Appendix 2, Figure A1 (underlined sequences). The two candidate Inr elements both fit the core sequence and are flanked on either side by pyrimidines. Interestingly, their positions would place the transcription start sites at positions 5663 & 5670 bp on the Suffolk PrP promoter, therefore only 3, and 1 bases away, respectively, from the positions mapped in previous studies (Westaway *et al.*, 1994b).

The Inr elements are perfectly conserved in the bovine PrP gene promoter (underlined sequences, Appendix 2, Figure A2.), suggesting that the transcriptional

start site of the PrP gene may be the same in these two species (Inoue *et al.*, 1997). Analysis of the mouse PrP gene promoter revealed the presence of three Inr elements (underlined sequences, Appendix 2, Figure A2), all of which are situated very close to potential transcription start sites as identified by Westaway *et al.* (1994a). In addition, a number of potential Inr elements were observed in the Syrian hamster, human and rat PrP gene promoters (underlined sequences, Appendix 2, Figure A2).

The ovine PrP gene promoter was also analysed for the presence of downstream promoter elements (DPEs) (Section 1.10.1) at approximately +30 bases from the potential transcription start sites/ Inr elements. The sequence of the DPE has been elucidated by a number of researchers and fits the broad consensus of (A/G/T)(G/C)(A/T)(C/T)(G/A/C)(T/C)G (Burke & Kadonaga, 1996, Burke & Kadonaga, 1997, Kadonaga, 2002, Kutach & Kadonaga, 2000). A single candidate DPE (bold text, Appendix 2, Figure A3) was found at position +31 from the first of the two potential ovine transcription start sites, although this sequence (AGAGCGT) varies in three positions (red text) from the DPE consensus (Burke & Kadonaga, 1997). However, this potential DPE sequence is perfectly conserved in the bovine PrP promoter, and as found in the ovine promoter it lies at position +31 bases from the first Inr element. Sequence analysis of the human promoter revealed the presence of two potential DPEs (GGTCCCC) and (AGCGCCG) at position +31 bases from the first and third Inr elements, respectively (bold text, Appendix 2, Figure A3). In addition, the rodent PrP promoters each contain a single conserved DPE sequence (AGGTAAG, bold text, Appendix 2, Figure A3), which varies at two positions from the consensus, and is located at position +35 (mouse), +30 (rat and Syrian hamster). A line up of the mammalian Inr and DPEs is presented in Appendix 2, Figure A3.

The location of the DPEs in the mammalian PrP promoters may indicate which Inr elements are functional in each promoter, and hence may indicate the location of the transcriptional start sites in these promoters. For example, the DPE in ruminants is located at position +31 bases from the first Inr element, therefore this first Inr element may be functional (Appendix 2, Figure A3). The same principal may be applied to the other mammalian promoters.

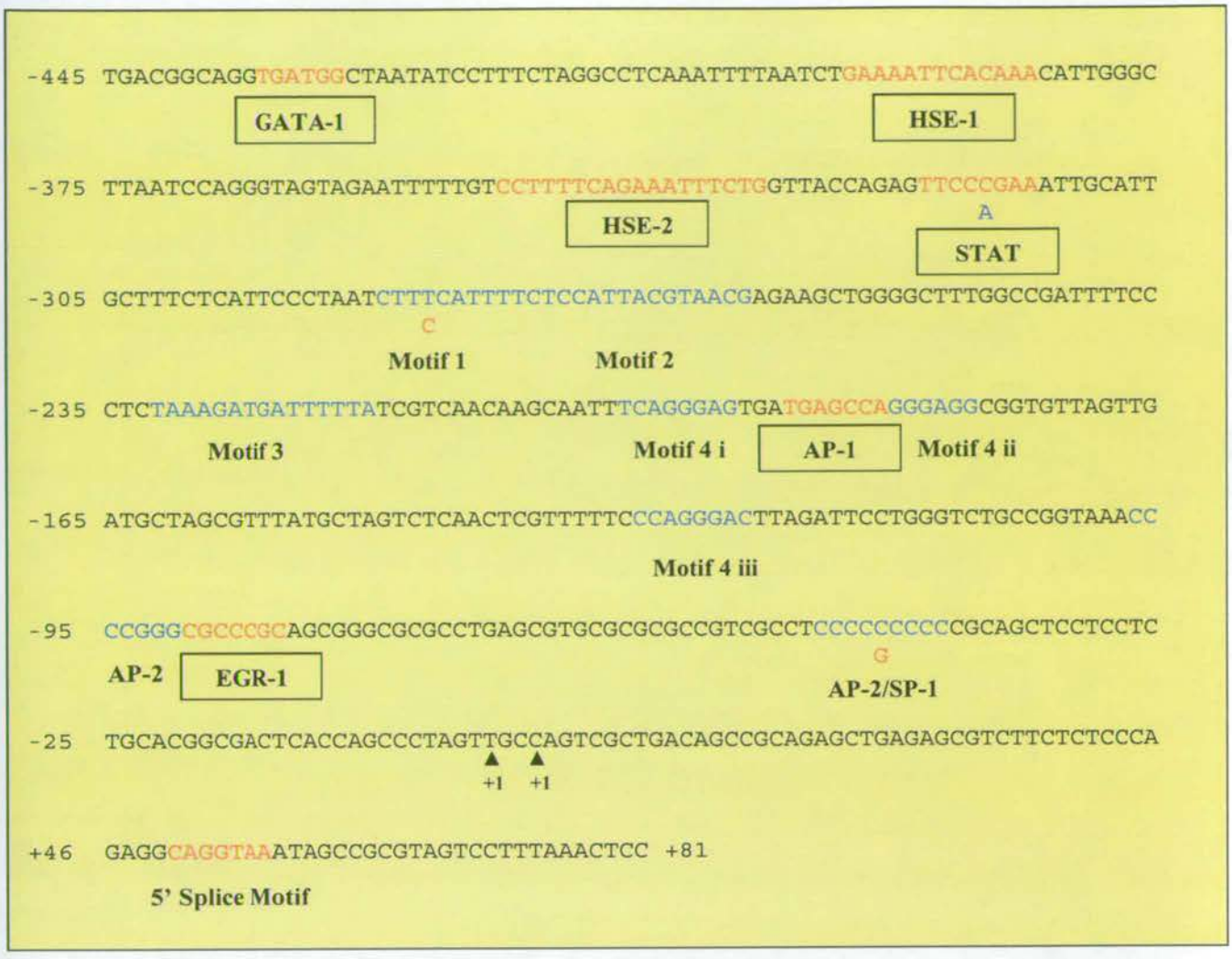


Figure 5.1 *Suffolk sheep PrP gene promoter transcription factor binding motifs*

Motifs 1-4 are conserved sequence elements found in ovine, murine, hamster, cattle and human PrP gene promoters, as identified by Westaway *et al.* (1994a) which may form binding sites for as yet unidentified transcription factors. Arrowheads indicate location of transcription start sites as experimentally shown by Westaway *et al.* (1994a). Suffolk sheep PrP gene promoter sequence from -445 to +81 bp, numbering based on the use of the 5' transcription start site (Genbank = U67922). Also highlighted are a potential binding site for the AP-2 transcription factor and a polymorphic AP-2/SP-1 site seen in Cheviot sheep (O'Neill *et al.*, 2003). Boxed factors are putative transcription factor binding motifs identified in this study and correspond to the sequence (indicated in red text) above the boxed title. Polymorphic base changes are indicated by base change (different colour) under the wild type (Suffolk, Genbank = U67922) sequence.

5.2.2 Analysis of binding to ovine PrP promoter AP-2 motifs

Factor binding to the upstream ovine AP-2 site and the downstream ovine AP-2 cluster motif, and its polymorphic variant previously identified by other researchers were analysed using gel shift assays carried out with nuclear extracts prepared from ovine cell cultures (sA80BR, pA80BR, IS120Cer & IS120Liv). Double stranded radiolabeled oligonucleotides (20 bp) were produced (Section 2.4.1 & 2.4.2) each of which contained the specific binding motif along with the native DNA flanking sequence (Tables 2.3 & 5.1 & Figure 5.1). Comparisons of mutated and wild type DNA sequences were made within the same batch of nuclear extract. In addition, all of the gel shift assays in this chapter were repeated on at least two occasions using different batches of nuclear extracts, unless stated otherwise.

Name	Abbreviation	DNA binding motif	Location
AP-1	AP-1	5'TGAGCCA'3	-190
Upstream AP-2	uAP-2	5'CCCCGGG'3	-97
Downstream AP-2 (Suffolk)	dAP-2s	5'CCCCCCCCC'3	-48
Downstream AP-2 (Cheviot)	dAP-2c	5'CCCCGCCCC'3	-48
EGR-1	EGR-1	5'CGCCCGC'3	-90
GATA-1	GATA-1	5'TGATGG'3	-434
HSE-1	HSE-1	5'GAAAATTCACAAA'3	-396
HSE-2	HSE-2	5'CCTTTTCAGAAATTTCTG'3	-349
Wild type motif 1	M1T	5'CTTTCATTTTCTCCA'3	-286
Variant motif 1	M1C	5'CTTCCATTTTCTCCA'3	-286
Ruminant motif 2	M2C	5'TTACGTAACG'3	-271
Non-ruminant motif 2	M2T	5'TTATGTAACG'3	-271
Motif 3	M3	5'TAAAGATGATTTTTA'3	-232
Motif 4 (i)	M4i	5'TCAGGGAG'3	-201
Motif 4 (ii)	M4ii	5'CCAGGGAGG'3	-186
Motif 4 (iii)	M4iii	5'CCAGGGAC'3	-130
Wild type STAT	STAT-C	5'TTCCCGAA'3	-321
Variant STAT	STAT-A	5'TTCCAGAA'3	-321

Table 5.1 *Ovine PrP promoter potential transcription factor binding motifs*

Details the putative transcription factor binding sites on the ovine PrP promoter. Location of each site is given relative to the 5' transcription start site as experimentally determined by Westaway *et al.* (1994a). Red text indicates the presence of a polymorphic or species specific base change.

5.2.2.1 *Characterisation of binding to the upstream ovine PrP promoter AP-2 motif*

The ovine PrP promoter contains a consensus binding motif for the AP-2 transcription factor (CCCCGGG) (upstream AP-2, -97 bp, Figure 5.1), and binding to this motif was investigated by gel shift assay using the uAP-2 oligonucleotide. A double stranded oligonucleotide containing this motif was produced (Section 2.4.1 & table 2.3), and radiolabeled with ^{32}P (Section 2.4.2). The uAP-2 oligonucleotide was incubated in gel shift assays (Section 2.4.6) with a variety of ovine cell culture nuclear extracts (IS120Cer, IS120Liv, sA80BR, pA80BR) (Section 2.4.3), and the results are shown in Figure 5.2.

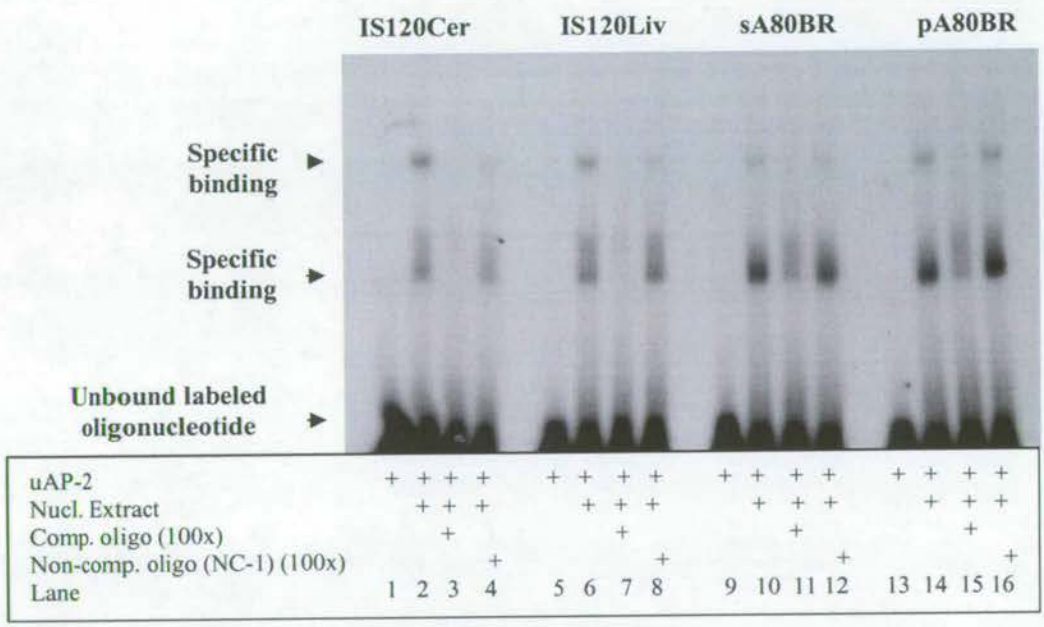


Figure 5.2 *Gel shift assay: upstream ovine AP-2 motif*

Gel shift assay indicating that the uAP-2 oligonucleotide is specifically bound by at least one transcription factor in all of the ovine cell line nuclear extracts tested. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
IS120Cer: nuclear extract from the IS120Cer (cerebellum) cell line
IS120Liv: nuclear extract from the IS120Liv (liver) cell line
sA80BR: nuclear extract from the sA80BR (scrapie susceptible) neuronal cell line
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

Two DNA-complexes of different mobilities were observed with the uAP-2 oligonucleotide with all of the ovine nuclear extracts tested (Lanes 2, 6, 10 & 14, Figure 5.2). These complexes were dissociated following the addition of 100 fold molar excess unlabelled competitor oligonucleotide, uAP-2 (Lanes 3, 7, 11 &12, Figure 5.2), however, they were not affected by the addition of 100 fold molar excess unlabelled non-competitor oligonucleotide, NC-1 (Table 2.3, Promega, UK) (Lanes 4, 8, 12 & 16, Figure 5.2), indicating that they were specific complexes. The factor binding to this motif is likely to be the transcription factor AP-2, and the different complexes may represent the monomeric (molecular weight of ~50 kDa) and dimeric (molecular weight of ~100 kDa) forms of the AP-2 protein (Lanes 2, 6, 10 & 14, Figure 5.2). A purified form of the murine AP-2 transcription factor was obtained from Promega, UK, and this factor was incubated in a gel shift assay (2.4.6) with a consensus AP-2 motif oligonucleotide (NC-2, Table 2.3, Promega, UK). The results of this gel shift assay were compared to that of the upstream ovine AP-2 motif gel shift assay (Figure 5.3). The mobility of the complex observed with the consensus AP-2 oligonucleotide (NC-2) and the purified murine AP-2 protein (Lanes 1-4, Figure 5.3) was similar to that observed with the larger complex of the upstream ovine AP-2 motif in the ovine cell culture nuclear extracts (Lanes 5-8, Figure 5.3). However, no equivalent of the lower band was seen with the purified murine AP-2 protein. These results indicate that it is likely that the factor binding to the ovine upstream AP-2 motif is AP-2 (Figure 5.3). The upstream AP-2 and EGR-1 binding motifs overlap each other, therefore the uAP-2 oligonucleotide also contains the entire EGR-1 binding site. (Figure 5.1). As such, the possibility that the lower band in Figure 5.2 could represent the binding of the 57 kDa EGR-1 protein cannot be ruled out.

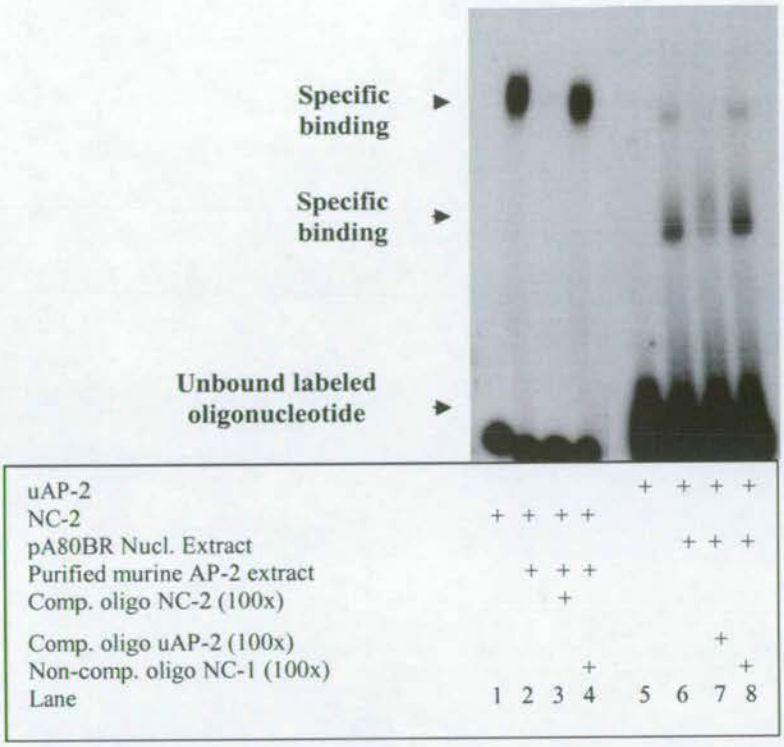


Figure 5.3 *Gel shift assay: Consensus AP-2 oligonucleotide with purified murine AP-2 protein compared to uAP-2 oligonucleotide with pA80BR nuclear extract (NE)*

Gel shift assay indicating that the uAP-2 oligonucleotide is specifically bound by at least one transcription factor (top band, lane 6) with a similar electrophoretic mobility to recombinant murine AP-2. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

5.2.2.2 *Characterisation of binding to the downstream ovine PrP promoter AP-2 motif cluster*

Compared with the published Suffolk PrP gene promoter, in most cases the Cheviot promoter contains a single base polymorphism of C to G, which lies within the downstream AP-2 motif cluster (-48 bp, Figure 5.4). This single base change has

a dramatic affect on the number of available AP-2 motifs within this cluster. Following the polymorphic change, five of the six AP-2 motifs in this AP-2 cluster are ablated, and a novel SP-1 motif is created (Figure 5.4). Therefore, the Cheviot PrP gene promoter has only one AP-2 motif and one SP-1 motif available (Figure 5.4). In contrast, the Suffolk PrP gene promoter has six overlapping AP-2 motifs available in a cluster and no SP-1 motif (Figure 5.4).

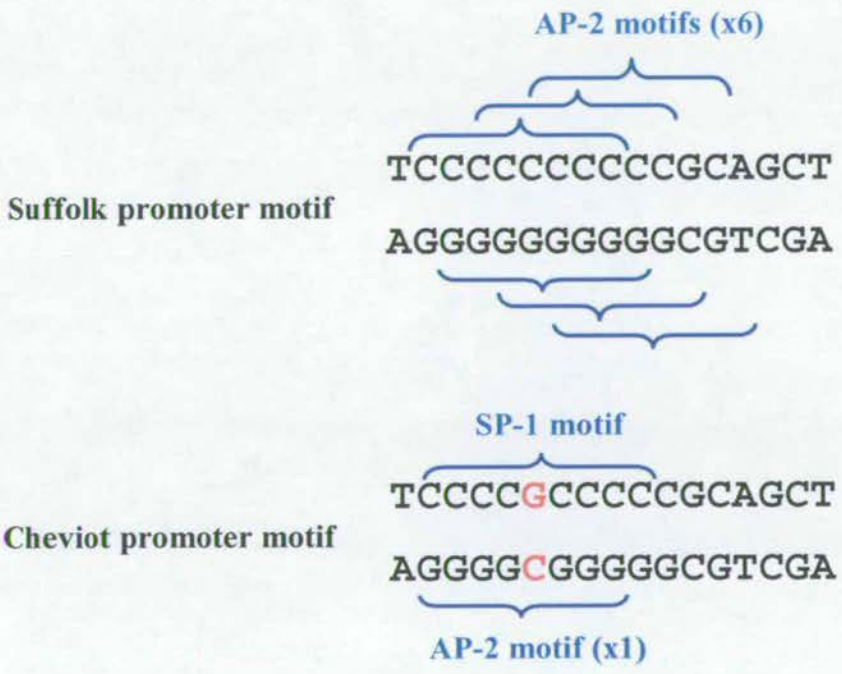


Figure 5.4 *Potential effect of the polymorphic change within the downstream ovine AP-2 motif cluster*

Diagram shows the Suffolk and Cheviot sheep polymorphic AP-2/SP-1 promoter motifs. The Cheviot sheep sequence contains a C to G polymorphic change at position 6 within this motif which appears to ablate five of the six overlapping AP-2 sites in this cluster motif, in addition this single base change creates a consensus SP-1 binding site. Sequences shown are of double stranded DNA from the Suffolk (Genbank = U67922) and Cheviot sheep promoters. Red bases indicate polymorphic base change.

Two double stranded oligonucleotides were prepared (Section 2.4.1 & table 2.3), one contained the Cheviot PrP promoter motif (dAP-2c, CCCCGCCCC), the other contained the Suffolk PrP promoter motif (dAP-2s, CCCCCCCCC) and both

were radiolabeled with ^{32}P (Section 2.4.2). Binding to the dAP-2c & dAP-2s oligonucleotides was analysed by gel shift assay (Section 2.4.6) with ovine pA80BR nuclear extracts (Section 2.4.3), and the results are shown in Figure 5.5.

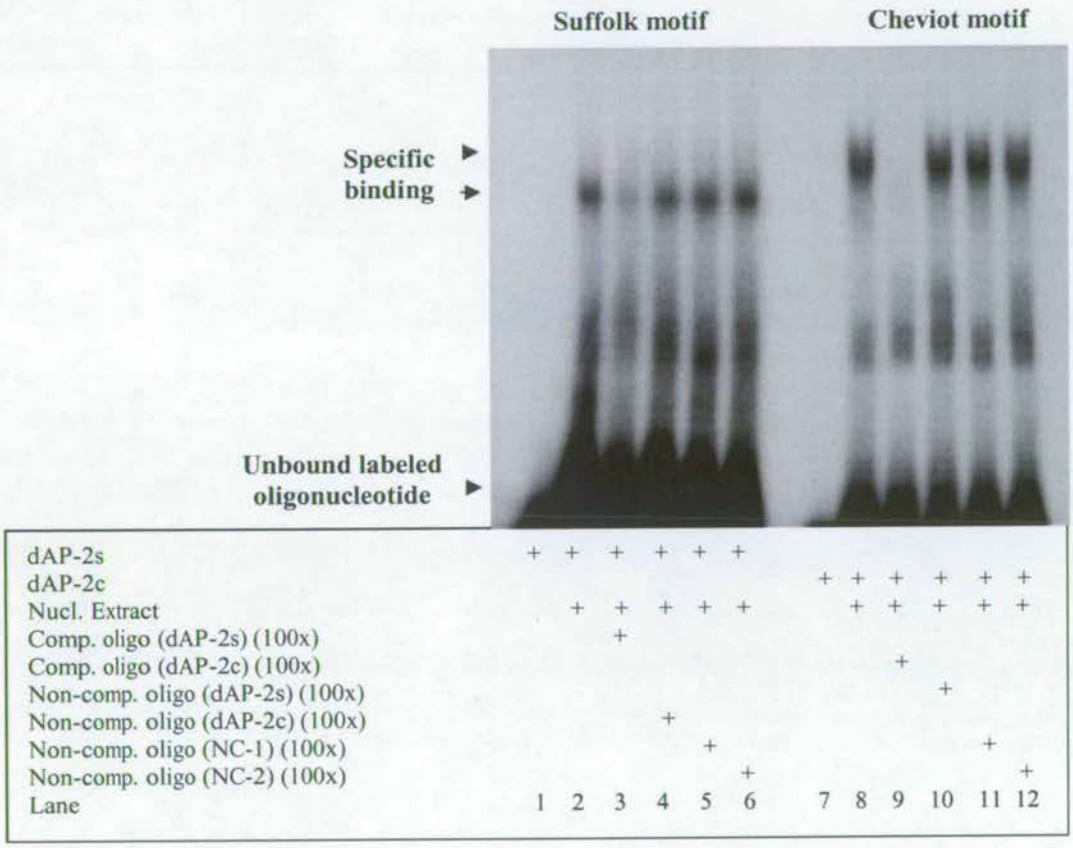


Figure 5.5 *Gel shift assay: polymorphic variants of the downstream ovine PrP promoter AP-2 motif cluster with pA80BR nuclear extract (NE)*

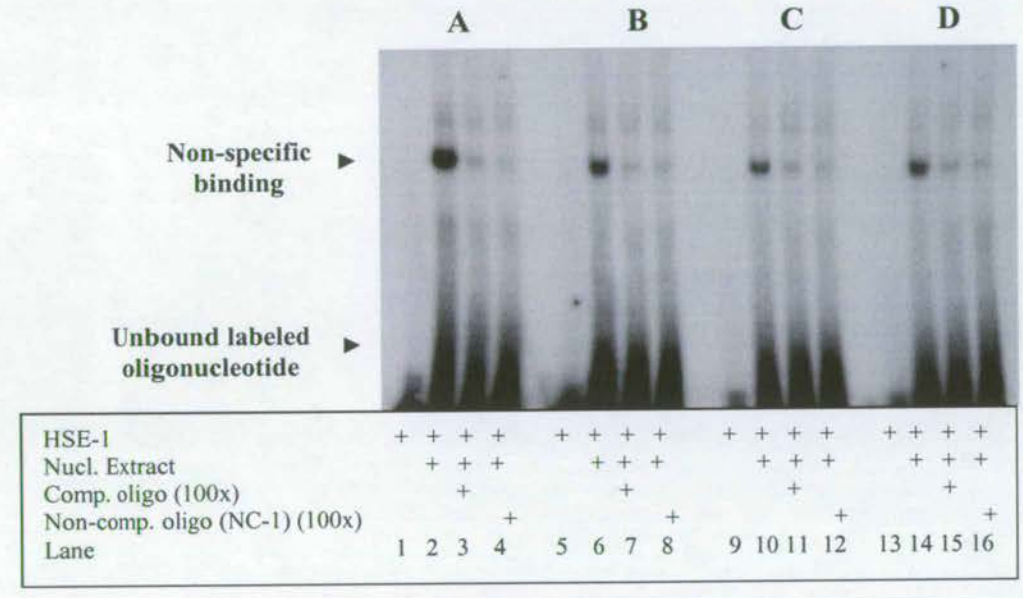
Gel shift assay indicating that the uAP-2s and uAP-2c oligonucleotides are specifically bound by transcription factors with different electrophoretic mobilities. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

Two DNA-complexes of different mobilities were observed with the two variants of the downstream ovine AP-2 binding motif in pA80BR nuclear extract (Suffolk & Cheviot, Figure 5.5). The complex observed with the dAP-2s oligonucleotide was not fully dissociated following the addition of 100 fold molar excess unlabelled competitor oligonucleotide, dAP-2s (Lane 3, Figure 5.5). However, the same complex was not affected by the addition of 100 fold molar excess unlabelled non-competitor oligonucleotides, dAP-2c, NC-1 & NC-2 (Lanes 4, 5, & 6, Figure 5.5), indicating that it was a specific complex. The complex observed with the dAP-2c oligonucleotide was fully dissociated following the addition of 100 fold molar excess unlabelled competitor oligonucleotide, dAP-2c (Lane 9, Figure 5.5), and was not affected by the addition of 100 fold molar excess unlabelled non-competitor oligonucleotides, dAP-2s, NC-1 & NC-2 (Lanes 10, 11 & 12, Figure 5.5) indicating that it was a specific complex. The dAP-2s and dAP-2c protein-DNA complexes were not competed out by the addition of each others unlabelled competitor oligonucleotides (Lanes 4 & 10, Figure 5.5) and this combined with the differing mobilities observed with the two complexes suggested that they are very likely to be different transcription factors. The Cheviot motif (Lanes 1-6, Figure 5.5) was bound by what appears to be a slightly larger complex than the Suffolk motif (Lanes 7-12, Figure 5.5), consistent with SP-1 (~106 kDa) binding to the Cheviot motif, and AP-2 (~100 kDa) binding to the Suffolk motif. This result is as expected, as the Cheviot motif contains a consensus SP-1 motif (CCCCGCCCC), whilst the Suffolk motif contains an AP-2 motif cluster (CCCCCCCCC) (Figure 5.5). This is further supported by studies by Nardelli, Gibson, Vesque *et al.* (1991) which showed that SP-1, which is a zinc finger DNA binding protein, binds to its DNA motif with three zinc fingers of two different types (type I & type II). Zinc fingers 1 & 3 are type II fingers, which recognise the CCC sequence on either end of the SP-1 motif, whilst zinc finger 2 is a type I finger, which recognises the CGC sequence at the centre of the SP-1 motif (Nardelli *et al.*, 1991). Therefore, the Suffolk AP-2 motif cluster would be unlikely to be bound by SP-1, as the centre zinc finger of SP-1 would not be able to bind. In contrast, SP-1 would be able to specifically recognise the Cheviot motif, due to the presence of the CGC sequence at its core.

5.2.3 Characterisation of binding to the ovine PrP promoter heat shock elements (HSE-1 and HSE-2)

The ovine PrP promoter contains two potential heat shock element (HSE-1 (-387 bp) & HSE-2(-340 bp)) binding sequences, to which the heat shock transcription factor (HSF) can bind (Figure 5.1). HSEs contain multiple copies of a repeating five base pair motif (nGAAn), usually arranged in alternating orientations (nGAAnnTTCnnGAAn) (Amin *et al.*, 1988, Fernandes, Xiao & Lis, 1994, Wu, 1995). A minimum of three repeats is required for the binding of HSF, which will bind cooperatively as a protein trimer to the HSE (Bonner, Ballou & Fackenthal, 1994). Each single repeat sequence can vary from the consensus of (nGAAn), for example (nAAAn) and (nGAGn) have been shown to be functional (Santoro, Johansson & Thiele, 1998). In addition, a functional HSE can tolerate up to a five base pair insertion between repeats, provided that the spacing and orientation of the repeats is maintained (Amin *et al.*, 1988, Wu, 1995). The ovine HSE-1 motif contains three alternate repeats of the (nGAAn) element, with just one base change from the consensus motif, marked in red text (nGAAnnTTCnnCAAn). The ovine HSE-2 motif also contains three alternating repeats, this time in a reverse direction, and with a one base insertion, marked in red text (nCTTnnnGAAnnTTCn). Therefore, it is possible that the ovine HSE motifs could be bound by HSF and may be functional. These sequences are conserved in the other mammalian PrP promoters analysed. For example, HSE-1 (TGAAATTCACAAA) is perfectly conserved in the bovine PrP promoter, and is also present in the human PrP promoter, but with one base change (TGAAATTCATAAA). HSE-2 (CCTTTTAGAAATTTCTG) is conserved in all of the mammalian PrP promoters, although there are some base changes between the different species. Expression of the HSF is regulated by cellular stress and heat shock. Once its expression is upregulated it binds to the HSE, and by doing so it promotes the expression of specific heat shock genes (Tanguay, 1988). In order to check the functionality of these HSE motifs in the ovine PrP promoter, oligonucleotides containing the ovine HSE-1 and HSE-2 motifs were prepared (Section 2.4.1 & table 2.3), and radiolabeled with ^{32}P (Section 2.4.2). Binding to these motifs was assessed with both normal, and a variety of heat shocked ovine pA80BR nuclear extracts (Section 2.4.3). In summary, nuclear extracts were

prepared from pA80BR cells that had been heat shocked at 42 °C for 30 minutes. Three sets of nuclear extracts were prepared, the first were heat shocked as above, and nuclear extracts were prepared immediately. Other cells were heat shocked and allowed to recover at 33 °C for one, or three hours prior to the preparation of nuclear extracts, in order to allow the cells time to activate the expression of HSF. The results of the gel shift binding assays of the HSE-1 oligonucleotide are shown in Figure 5.6.



KEY: A = non-heat shocked pA80BR nuclear extract (NE); B = heat shocked pA80BR NE, no recovery time; C = heat shocked pA80BR NE, 1 hour recovery time; D = heat shocked pA80BR NE, 3 hour recovery time.

Figure 5.6 *Gel shift assay: ovine HSE-1 oligonucleotide*

Gel shift assay indicating that the ovine HSE-1 oligonucleotide is not specifically bound by transcription factors in either heat shocked or non-heat shocked pA80BR cell line nuclear extracts. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

Binding was observed to the HSE-1 & HSE-2 elements with both the normal and the heat shocked pA80BR cell culture nuclear extracts (only HSE-1 shown, Figure 5.6), however, the binding was competed out by both 100 fold molar excess unlabelled competitor (HSE-1, Lanes 3, 7, 11 & 12, Figure 5.6) and 100 fold molar excess unlabelled non-competitor oligonucleotide (NC-1, Lanes 4, 8, 12 & 16, Figure 5.6), indicating that it was not specific. No difference in binding was observed between the non-heat shocked nuclear extract (Set A, Figure 5.6), the nuclear extracts which were prepared from cells which had been allowed to recover for 1 or 3 hours (Sets B & C, Figure 5.6), following heat shock and those that were processed immediately after heat shock treatment (Set B, Figure 5.6). These results suggested that heat shock treatment had no effect on binding to the ovine HSE-1 and HSE-2 motifs.

5.2.4 Characterisation of binding to the ovine PrP promoter AP-1, EGR-1 & GATA-1 transcription factor binding motifs

A potential AP-1 binding motif (-190 bp, ATGAGCCAG) was identified in the ovine PrP promoter, located between motifs 4 (i) and (ii) (Figure 5.1). This motif varies by one base (marked in red text) from the AP-1 consensus binding motif of (A/T)T(G/T)(A/C)(G/C)TCA(G/C/A). AP-1 is a dimeric transcription factor, which consists of two monomers of the Jun protein in a homodimeric complex, or one Fos & one Jun monomer in a heterodimeric complex (Bohmann, Bos, Admon *et al.*, 1987, Landschulz, Johnson & McKnight, 1988, Landschulz, Johnson & McKnight, 1989, Turner & Tjian, 1989). AP-1 has been shown to tolerate single base changes to its consensus binding motif, although multiple sequence changes may abolish binding (Risse, Jooss, Neuberg *et al.*, 1989). In addition, slight variations to the consensus motif may allow the binding of other members of the leucine zipper family to bind, such as the activating transcription factor (ATF) protein family, thus forming more complex hetero-dimers (Angel & Karin, 1991). The bovine PrP promoter contains a similar sequence at the same location (ATGAGCCGG). However, this motif varies at two positions from the AP-1 consensus, and is therefore less likely to form a binding motif for AP-1, although this was not tested in this study. In addition, the rodent and human PrP promoters all contain at least one

consensus AP-1 motif (Baybutt & Manson, 1997, Funke-Kaiser *et al.*, 2001, Saeki *et al.*, 1996). The presence of EGR-1 (-90 bp) & GATA-1 (-429 bp) motifs in the ovine PrP gene promoter was interesting as both of these factors have been shown to interact with SP-1 to regulate the expression of specific genes (Ebert & Wong, 1995, Fischer, Haese & Nowock, 1993, Sukhatme *et al.*, 1988, Tsai *et al.*, 1989). Oligonucleotides containing the ovine AP-1, EGR-1 & GATA-1 motifs were prepared (Section 2.4.1 & table 2.3), labeled with ^{32}P (Section 2.4.2) and incubated in gel shift assays (Section 2.4.6) with ovine pA80BR nuclear extracts (Section 2.4.3). The results of these gel shift assays are shown in Figure 5.7.

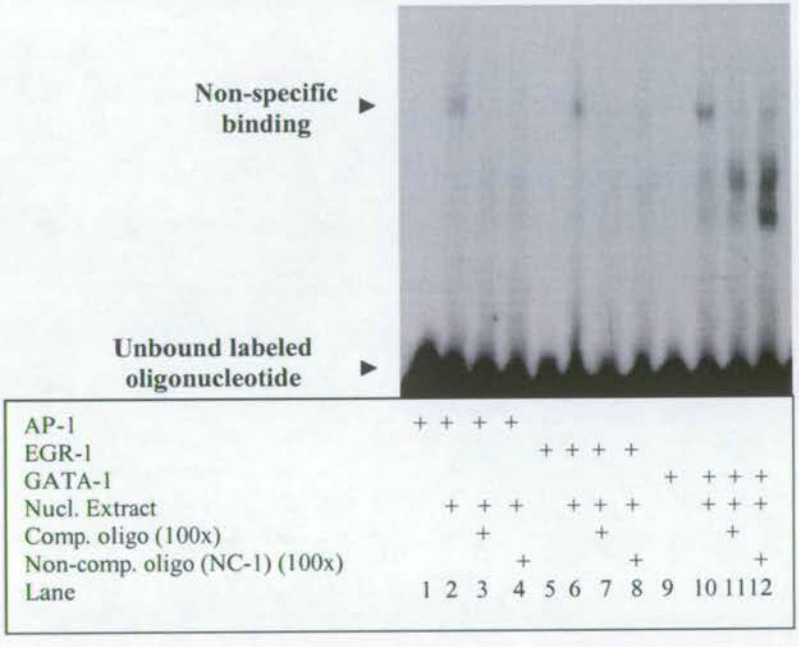


Figure 5.7 *Gel shift assay: ovine AP-1, EGR-1 & GATA-1 oligonucleotides with pA80BR nuclear extract (NE)*

Gel shift assay indicating that the ovine AP-1, EGR-1 & GATA-1 oligonucleotides are not specifically bound by transcription factors in the ovine pA80BR cell line nuclear extract. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

Very faint binding was observed to the AP-1, EGR-1 and GATA-1 oligonucleotides with the ovine pA80BR nuclear extracts (Lanes 2, 6 & 10, Figure 5.7). However, the binding was competed out by both 100 fold molar excess unlabelled competitor (Lanes 3, 7 & 11, Figure 5.7) and 100 fold molar excess unlabelled non-competitor oligonucleotide (Lanes 4, 8, 12, Figure 5.7), indicating that it was not specific. In addition, binding was observed in the GATA-1 gel shift assay after the addition of the 100 fold molar excess unlabelled competitor oligonucleotide (Lane 11, Figure 5.7), indicating the possibility of an interaction between the labeled complex and the unlabelled competitor oligonucleotide. However, this binding was also observed following the addition of 100 fold molar excess non-competitor oligonucleotide, NC-1 (Lane 12, Figure 5.7), indicating that this binding was not relevant. Finally, as no specific binding was observed to the EGR-1 oligonucleotide, which also contains a section of the uAP-2 oligonucleotide, but not the entire upstream AP-2 binding motif, it is reasonable to assume that the lower band observed with the uAP-2 oligonucleotide (Figure 5.2) could be that of the AP-2 monomer and not EGR-1.

5.2.5 Characterisation of binding to the ovine PrP promoter polymorphic STAT motif

The ovine PrP promoter contains a C to A polymorphism (-317 bp), which is associated with the positive line in the NPU Cheviot flock and is present in the human and bovine PrP promoters (O'Neill *et al.*, 2003) (Figure 5.1). Sequence analysis of this polymorphism revealed that the wild type sequence (-321 bp, TTCCCGAAA) closely fits the consensus binding motif (TTCCCGGAA) for the STAT family of transcription factors, with only one base change (Yan *et al.*, 1996). In contrast, the polymorphic change of C to A creates a variant STAT motif (-321 bp, TTCCAGAAA) with two base changes from the STAT consensus motif. Similar STAT motifs are present in the rodent PrP promoters, but with some sequence variations (rat = TTCCAGATG; mouse = TTCAACGAT; Syrian hamster = TTCAGCAAT). Interestingly, different STAT family proteins have been shown to bind to slightly different STAT binding motifs, for example STAT-1, 3 & 4 favour a G or C at position 5, whilst STAT-5 & 6 favour an A or T at the same position (Ihle,

1996). It is therefore possible that different STAT proteins could bind to the two polymorphic variations of the ovine STAT motif. Oligonucleotides were produced (Sections 2.4.1 & 2.4.2), which contained the two variations of this polymorphic motif (Table 2.3). Binding to the two oligonucleotides (STAT-C and STAT-A) was investigated using gel shift assays (Section 2.4.6), carried out on only one occasion, with the sA80BR, pA80BR, IS120Cer & IS120Liv ovine cell culture nuclear extracts with the same result (Section 2.4.3) (only pA80BR shown, Figure 5.8).

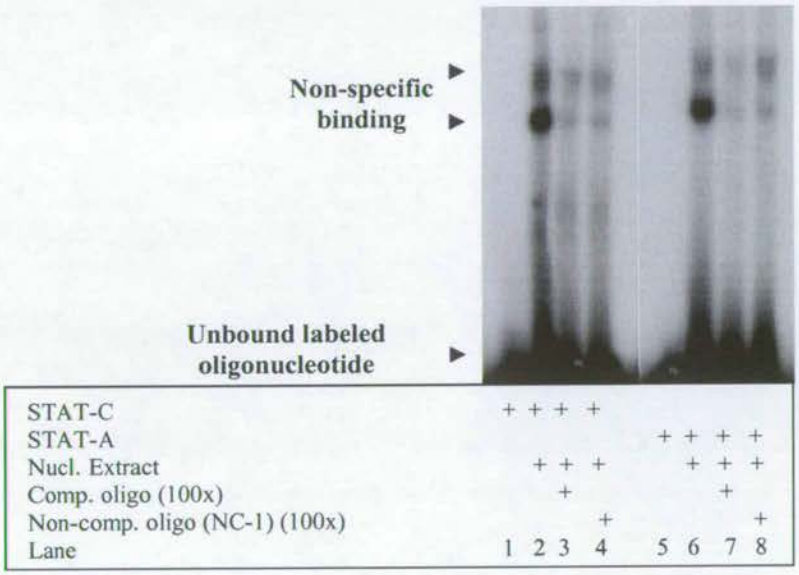


Figure 5.8 *Gel shift assay: STAT-C & STAT-A oligonucleotides with pA80BR nuclear extract (NE)*

Gel shift assay indicating that the ovine polymorphic STAT-C & STAT-A oligonucleotides are not specifically bound by transcription factors in the ovine pA80BR cell line nuclear extract. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

As can be seen from Figure 5.8, the results with the STAT motif oligonucleotides were inconclusive. Two complexes were observed with both of the STAT oligonucleotides (Lanes 2 & 6, Figure 5.8). Both of the complexes observed with the STAT-C oligonucleotide were competed out by the addition of 100 fold molar excess unlabelled competitor and non-competitor (NC-1) oligonucleotides, indicating that this represented non-specific binding (Lanes 3 & 4, Figure 5.8). The smaller complex observed with the STAT-A oligonucleotide was competed out by the addition of 100 fold molar excess unlabelled competitor and non-competitor (NC-1) oligonucleotides (Lanes 7 & 8, Figure 5.8). In contrast, the larger complex observed with the STAT-A motif, was competed out by the addition of 100 fold molar excess unlabelled competitor oligonucleotide (Lane 7, Figure 5.8), but was not competed out by the addition of 100 fold molar excess unlabelled non-competitor (NC-1) oligonucleotide (Lane 8, Figure 5.8), indicating that this binding may have been specific, however further studies are required to confirm this.

5.3 Discussion

Sequence analysis of the ovine PrP gene promoter revealed the presence of a number of transcription factor binding motifs, which had not been previously identified. All of these motifs were found within a section of DNA stretching to approximately 500 bp upstream from the potential transcription start sites identified by Westaway *et al.* (1994b). These included potential binding motifs for the HSF, STAT family, AP-1, EGR-1 & GATA-1 transcription factors. The biochemical functionality of these motifs, along with those for upstream ovine AP-2 motif, and the downstream AP-2 motif cluster, were analysed by gel shift assay and binding to the polymorphic variations of these motifs, where appropriate, was assessed.

All of the mammalian PrP promoters analysed contained Inr elements, and in most cases these were located at, or very close to the positions of previously identified transcription start sites (Appendix 2, Figures A1 & A2). The conserved nature of these Inr elements indicates that they may be functional, and the position of the A_{+1} residues in these sites may constitute the transcription start sites of the respective PrP genes.

Transcription in TATA-less promoters, such as the PrP gene promoter, is initiated by the binding of a complex of general transcription factors (GTFs) termed TFIID, comprised of TBP and TBP-associated factors or TAF_{II}s (Conaway & Conaway, 1993, Green, 2000, Ptashne, 1988, Verrijzer & Tjian, 1996). TFIID binds specifically to an Inr element ($PyPyA_{+1}N(T/A)PyPy$), where A_{+1} is the transcriptional start site (Lo & Smale, 1996, Smale, 1997). In addition, the binding of the TFIID complex is aided in some TATA-less promoters by the presence of a DPE (Burke & Kadonaga, 1996, Burke & Kadonaga, 1997, Kadonaga, 2002, Kutach & Kadonaga, 2000, Smale, 1997). The DPE sequence acts as a specific binding motif for a GTF termed TAF_I150, and in promoters, which contain a DPE, a specific TBP-DNA interaction may not be required (Smale, 1997). Instead the Inr element is bound specifically by a GTF termed $TAF_{II}250$ in association with TBP, and the DPE is bound by TAF_I150 (Smale, 1997) (Figure 5.9). $TAF_{II}250$ and TAF_I150, each bound to their respective promoter elements, are then able to interact with each other and TBP to form an effective docking platform for RNA polymerase II (Smale, 1997) (Figure 5.9).

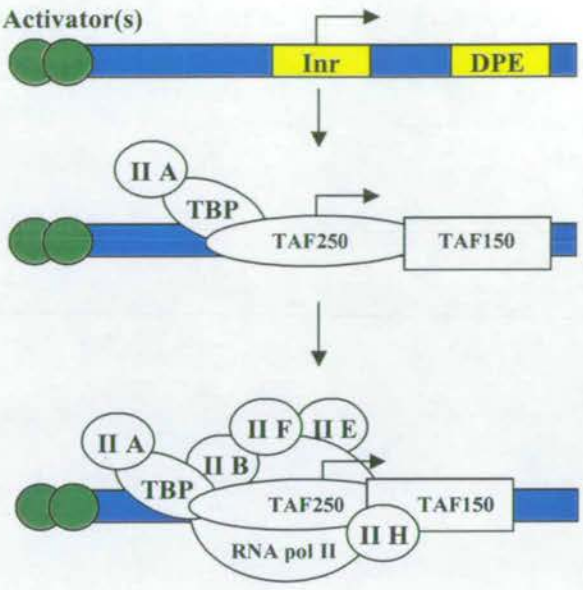


Figure 5.9 *Transcription initiation in TATA-less eukaryotic promoters with a DPE*

Diagram shows the potential interactions of general transcription factors (GTFs) on a promoter sequence that contains an initiator (Inr) element and a downstream promoter element (DPE). TBP = TATA binding protein, RNA pol. II = RNA polymerase II, IIA – IIH = GTFs and TAF150 & TAF250 = TBP associated factors.

Conserved downstream promoter elements (DPEs) were also found in all of the mammalian PrP promoters, located between +30 to +35 bases from the respective Inr elements. The discovery of conserved DPEs may help to further pinpoint the location of the actual transcription start sites in the PrP promoters, as most of the promoters analysed contained more than one Inr element (Appendix, Figure A3). For example, in ruminants and mice it appears that the first Inr element may be functional, as this Inr has a DPE located at approximately +30 bases downstream (Appendix 2, Figure A3). In the rat and hamster PrP promoters, the second Inr site may be functional, as in these species the DPE is located at +30 bases downstream from this Inr element (Appendix 2, Figure A3). Finally, the human PrP promoter

contains two DPEs, each located at approximately +30 bases downstream of an Inr element, indicating that either could be functional in this promoter.

This study has resulted in the identification of potential Inr elements and DPEs, located at what may be functional positions, in all of the PrP promoter sequences examined. These elements are conserved to a certain extent in the mammalian species tested, although some sequence variations are present (Appendix 2, Figure A3). Further studies will be necessary to characterise any binding to these elements in gel shift and super-shift assays, and analysis of the sequence variations may provide further clues as to the nature of PrP gene expression regulation in the different mammalian species.

The binding of TFIID to the Inr element may occur cooperatively with the transcription factors AP-2 & SP-1 (Laity *et al.*, 2001). As such AP-2 & SP-1 binding motifs are commonly found in TATA-less promoters (Hilton & Wang, 2003, Kadonaga *et al.*, 1987, Laity *et al.*, 2001, Mavrothalassitis, Watson & Papas, 1990, Philipsen & Suske, 1999, Salbaum *et al.*, 1989, Weis & Reinberg, 1997, Zhou & Chiang, 2002). All of the mammalian PrP promoters, with the exception of the ovine promoter, contain either two (human) or three (bovine & rodent) SP-1 motifs, located close to the 3' end of the promoter. In addition, all of the mammalian PrP promoters analysed contain varying numbers of AP-2 binding motifs. The Cheviot PrP promoter contains a single SP-1 motif, and this is likely to be the wild type situation, due to the conservation of SP-1 motif(s) in the other mammalian promoters. In contrast, the Suffolk PrP promoter does not contain a SP-1 motif, and instead contains an AP-2 binding motif cluster in the same location (Figure 5.4). Transcriptional initiation of the Cheviot, human, bovine & rodent PrP genes may therefore be regulated by a combination of SP-1 and AP-2 transcription factors. Whilst transcriptional initiation in the Suffolk PrP gene may be regulated by the binding of the AP-2 transcription factor. It is unclear what effect this differential regulation would have on PrP gene expression between Cheviot and Suffolk sheep, but it is certainly worthy of further investigation.

5.3.1 Upstream ovine AP-2 motif

Analysis of the upstream ovine AP-2 motif (Figure 5.1), showed that an oligonucleotide containing this sequence (uAP-2) was specifically bound by a nuclear protein factor present in all of the ovine cell line nuclear extracts tested (Figure 5.2). This factor is likely to be AP-2 and a comparison of this complex with a gel shift of a recombinant murine AP-2 protein with a consensus AP-2 oligonucleotide (NC-2) showed that the two complexes had very similar mobilities. However, a gel super-shift assay with an anti-AP-2 antibody could be used to confirm that this factor is AP-2.

The AP-2 transcription factor can act as either a repressor or an activator of transcription, depending on the promoter and the cellular context of its target gene (Roberts & Green, 1995). AP-2 may compete with other transcription factors for the sole occupation of a single binding motif, and this may contribute to the tissue-specific expression of target genes (Courtois, Lafontaine, Lemaigre *et al.*, 1990, Jiang, DeFrances, Machen *et al.*, 2000, Mercurio & Karin, 1989, Ren & Liao, 2001). It is unclear what role AP-2 may play in the regulation of PrP gene expression. However, deletion of a region of DNA containing the upstream AP-2 motif from the ovine PrP promoter linked to a CAT reporter gene, resulted in a considerable reduction in CAT activity in transiently transfected ovine sA80BR cells (O'Neill *et al.*, 2003). This region of the ovine PrP promoter also contains binding motifs for EGR-1, and motif 4 (iii), however, binding assays carried out in this study have shown that these two motifs are unlikely to be functional in the cell cultures used (EGR-1, Figure 5.7; motif 4 (iii), Chapter 6). In addition, the deletion of a region containing an AP-2 motif in the human and rat PrP gene promoters, results in a significant reduction in reporter gene expression in transiently transfected human and rat cells, respectively (Funke-Kaiser *et al.*, 2001, Saeki *et al.*, 1996). Therefore, the PrP promoter AP-2 motif appears to be functional and its deletion results in a loss of promoter activity in ovine, rat and human cells. This may indicate a role for the AP-2 transcription factor in transcriptional activation of the ovine PrP gene, although its actual role could be much more complex.

5.3.2 Downstream ovine AP-2 motif cluster

Analysis of the Suffolk sheep downstream ovine AP-2 motif cluster (dAP-2s) and the Cheviot variant of this motif (dAP-2c), revealed that the two variants are bound by proteins with different electrophoretic mobilities in ovine pA80BR nuclear extract (Figures 5.1, 5.4 & 5.5). However, in order to confirm these results it would be necessary to carry out super-shift assays with anti-AP-2 and anti-SP-1 antibodies, respectively. The transcription factors SP-1 and AP-2 have both been shown to activate the transcription of specific genes, and they may both play important roles in the binding of RNA polymerase II to the promoter (Kadonaga *et al.*, 1987, Pugh & Tjian, 1990, Zhou & Chiang, 2002). SP-1 and AP-2 are transcription factors, which bind specifically to GC boxes within gene promoter regions (Kadonaga *et al.*, 1987). SP-1 has been shown to interact with other nuclear factors, such as Yin Yang 1 (YY1), TBP, and the TBP associated factors $TAF_{II}110/TAF_{II}130$ and $TAF_{II}55$ (Chiang & Roeder, 1995, Emili, Greenblatt & Ingles, 1994, Hoey *et al.*, 1993, Lee, Galvin & Shi, 1993, Seto *et al.*, 1993, Tanese, Saluja, Vassallo *et al.*, 1996). SP-1 is an important factor in the regulation of transcriptional initiation from TATA-less promoters, and it is thought that it acts as a tethering moiety, to recruit the basal transcription complex to TATA-less promoters (Pugh & Tjian, 1990). However, SP-1 is not vital for transcriptional initiation from TATA-less promoters, and AP-2 may also be able to fulfill a similar role (Zhou & Chiang, 2002). Although SP-1 has defined roles in transcriptional activation, it has been shown to act as a repressor of transcription, by recruiting histone deacetylase activity (Doetzlhofer, Rotheneder, Lagger *et al.*, 1999). Finally, SP-1 may be involved in the tissue specific expression of its target genes by competing for the sole occupation of GC boxes with other factors, which recognise the same consensus motifs, such as SP-3, SP-4 and AP-2 (Kwon, Kim, Edenberg *et al.*, 1999, Suske, 1999).

It is still not known how the binding of either AP-2, or SP-1 to the ovine PrP gene promoter would affect gene expression. Deletion of the first of three SP-1 motifs in the bovine PrP gene promoter, linked to a CAT reporter gene resulted in a reduction in CAT activity in transiently transfected bovine fibroblast (CK-1) cells (Inoue *et al.*, 1997). In addition, deletion of the second SP-1 motif in the rat PrP promoter linked to a luciferase reporter gene, resulted in a reduction in luciferase

activity in transiently transfected rat pheochromocytoma (PC12) and glioma (C6) cell lines (Saeki *et al.*, 1996). Finally, studies by Baybutt & Manson (1997) showed that the first 80 bases upstream of the mouse PrP gene promoter transcription start site, which contains binding motifs for SP-1, AP-2 and AP-1, are required for full promoter activity. These results indicate that SP-1 may play an important role in the activation of PrP gene expression, at least in mice, rats and cattle, although its role in the regulation of ovine PrP gene expression is still to be elucidated.

Studies by O'Neill *et al.* (2003) have indicated that the upstream ovine AP-2 motif may activate ovine PrP gene expression. However, this activating role may not necessarily apply to the downstream AP-2 motif cluster, as the activation observed at the upstream motif could be triggered by its specific location within the promoter. The significance of the downstream ovine AP-2 motif cluster, consisting of six overlapping AP-2 motifs, in the Suffolk PrP promoter is unclear (Figures 5.4 & 5.5). However, the presence of AP-2 clusters within a variety of promoters has been noted by other researchers (Imagawa, Chiu & Karin, 1987, Novikov & Kamps, 2001, Shachaf, Skorecki & Tzukerman, 2000, Terzano, Flora, Clementi *et al.*, 2000). The mouse PrP promoter also contains an AP-2 motif cluster, and polymorphisms within this cluster, alter the number of AP-2 binding motifs available in different breeds of mice. For example, SV and L129 mice have four overlapping AP-2 motifs, VL mice have six motifs, and RIII mice have only one AP-2 motif at the same location (Baybutt & Manson, 1997). Interestingly, Imagawa, Chui & Karin (1987) showed that a cluster of five overlapping AP-2 motifs acted as an efficient cell-type specific enhancer element. In contrast, AP-2 has been shown to have a role in transcriptional repression, by competing with activating transcription factors for the same binding motif (Getman, Mutero, Inoue *et al.*, 1995, Jiang *et al.*, 2000, Ren & Liao, 2001). Therefore, these AP-2 clusters could potentially be acting as binding blocks, preventing the binding of other factors to an overlapping, or nearby motif. Further studies are required to investigate the role of AP-2 motif clusters in the murine and ovine PrP gene promoters. For example, binding activity to the murine polymorphic AP-2 motif cluster may reveal breed specific differential binding to these elements and may provide further insights into the role of these motifs in the regulation of PrP gene expression.

5.3.3 Ovine HSE-1 & HSE-2

The ovine PrP promoter contains two potential heat shock elements (HSE-1 & HSE-2, Figure 5.1) which could act as binding motifs for the HSF (Amin *et al.*, 1988, Wu, 1995). HSF is an inducible transcription factor which binds to the HSE in the promoters of heat shock genes (Morimoto, 1993). The expression of HSF is activated in response to cellular stress, caused by elevated temperatures or a variety of chemical and physiological stresses, and this is known as the heat shock response (Amin *et al.*, 1988, Wu, 1995). Shyu, Harn, Saeki *et al.* (2002) identified two HSEs further upstream in the rat PrP promoter (-680 bp & -1653 bp). The authors showed that both motifs were occupied by HSF in gel shift and super-shift assays carried out in nuclear extracts prepared from heat shocked human NT-2 cells (NTERA-2, pleuripotent, embryonal carcinoma cells), whilst only slight binding was observed in nuclear extracts prepared from non-heat shocked cells (Shyu *et al.*, 2002). The human β-amyloid precursor protein gene promoter also contains a single HSE, and specific binding by HSF was observed to an oligonucleotide containing this motif in gel shift and super-shift assays performed in nuclear extracts prepared from heat shocked human cells (Dewji & Do, 1996, Dewji, Do & Bayney, 1995). Binding to the ovine HSE-1 & HSE-2 oligonucleotides was assessed with nuclear extracts prepared from normal, and heat shocked pA80BR ovine cultured cells (Figure 5.6). The results showed that the ovine HSE motif oligonucleotides are not specifically bound by factors present in nuclear extracts prepared from pA80BR cells cultured, in either normal cellular conditions, or following heat shock treatment (Figure 5.6). However, the cell cultures used for the characterisation of the ovine PrP promoter HSEs (pA80BR) are normally cultured at 33 °C, and were heat shocked at 42 °C in these studies. It is possible that an increase of 9 °C may have had detrimental effects on these cells, and this may explain the lack of binding observed to the ovine HSEs. It may therefore be prudent to repeat these studies by heat shocking the pA80BR cells at 37 °C (a 4 °C increase), alternatively these studies could be repeated by heat shocking the IS120Cer cell line at 42 °C, as these are normally cultured at 37 °C.

5.3.4 Ovine AP-1, GATA-1 & EGR-1 motifs

The presence of an AP-1 motif on the ovine PrP promoter is very interesting, as the rodent and human PrP promoters all contain at least one consensus AP-1 motif. However, no specific binding was observed to the AP-1 oligonucleotide in gel shift assays carried out with nuclear extracts prepared from the ovine pA80BR cell culture (Figure 5.7). These results show that this motif is not functional in this system, at least in normal cellular conditions, as tested in this study. It may be the case that transcription of the ovine PrP promoter is not regulated by AP-1, however, the role of AP-1 in the other mammalian promoters remains unclear. In addition, no specific binding was observed to the GATA-1 and EGR-1 oligonucleotides with the ovine pA80BR nuclear extracts (Figure 5.7). The results show that these motifs are not functional in this system, however, binding of these factors could be activated by specific cellular stimuli, that may not have been replicated accurately in these studies.

5.3.5 Ovine PrP promoter STAT motif

No specific binding was observed to the STAT-C oligonucleotide, however, specific binding may have occurred with the STAT-A oligonucleotide with the pA80BR nuclear extract (Figure 5.8). However, this result would have to be repeated and could be combined with a gel super-shift assay using an anti-STAT antibody to identify the bound factor. The STAT family of transcription factors, are latent transcription factors, which are found in the cytoplasm of resting cells, and are thought to be involved in the transcriptional activation of a variety of genes (Darnell, 1997, Levy & Darnell, 2002). In response to a variety of stimuli, STAT proteins become phosphorylated, this induces them to form homo-dimers, which are able to translocate to the nucleus, where they are able to function as transcriptional activators (Darnell, 1997, Levy & Darnell, 2002). Therefore, binding to the ovine PrP promoter STAT motifs may occur only in response to specific stimuli, which have not been replicated in this system. Interestingly, the expression of STAT proteins is regulated by the cellular levels of specific cytokines, such as interleukin-2 (IL-2), IL-6, IL-10, IL-12, IL-4, interferon-γ (IFN-γ) and IFNα/IFNβ (Ihle, 1996,

Lackmann, Harpur, Oates *et al.*, 1998, Takeda & Akira, 2000), and the expression of proinflammatory cytokines, like IL-6 appear to be elevated in the brains of scrapie infected mice (Kim, Ju, Choi *et al.*, 1999). The ovine PrP promoter STAT motifs may therefore be occupied by disease specific factors, and it would be interesting to analyse binding to the ovine PrP promoter STAT motifs in nuclear extracts prepared from scrapie infected brain tissue. It would be very interesting to analyse binding to the ovine PrP promoter STAT motifs with nuclear extracts prepared from cells, which have been treated with the cytokine, IFN-γ, as this factor has been shown to induce the nuclear translocation of STAT proteins (Darnell, 1997, Lackmann *et al.*, 1998, Leonard & O'Shea, 1998, Schindler & Strehlow, 2000). In addition, it is possible that the observed binding to the STAT-A oligonucleotide could constitute the binding of a STAT factor to this motif, and that this binding does not occur to the wild type STAT motif. This may indicate that binding only occurs to the variant STAT motif within the ovine PrP promoter, which is associated with the positive line (scrapie susceptible) in the NPU Cheviot flock. The functionality of the ovine PrP promoter STAT motifs remains unclear, however, further studies into these motifs are necessary, and could provide insights into their role in the regulation of PrP gene expression, and possibly in scrapie susceptibility.

5.3.6 Conclusions

In summary, the results observed in this study have identified potential transcription factor binding motifs within the ovine PrP promoter. Gel shift assays showed that the upstream ovine AP-2 motif was specifically bound by a protein factor present in the ovine cell culture nuclear extracts. The downstream ovine AP-2 motif was also bound specifically and the C to G polymorphic change within this site resulted in the binding of what appeared to be a completely different factor to this element. Two potential Inr elements were identified at the 3' end of the ovine PrP promoter, which mapped potential transcription start sites to within 1-3 bases of those identified by other researchers. Potential Inr elements were identified within all of the other mammalian PrP promoters and where the information was available these sites were found to lie within a few bases of previously published transcription start sites. In addition, putative DPEs were identified at approximately +30 bases

from selected Inr elements within all of the mammalian PrP promoters analysed. Furthermore, the positioning of the Inr elements and DPEs indicated the potential location of transcription start sites within all of the mammalian PrP promoters. The worked carried out during this study has demonstrated specific binding to selected transcription factor motifs within the ovine PrP promoter. It has also shown that at least one polymorphic change within this sequence results in differential binding to this site. This study has demonstrated the functionality of a number of transcription elements within the ovine PrP promoter and has provided further insights into the role of the promoter region in the regulation of PrP gene expression.

Chapter 6: Characterisation of motifs 1-4 in the ovine PrP gene promoter region

6.1 Introduction

Four DNA sequence motifs have been identified in the ruminant, rodent and human PrP promoters based upon their conserved sequence homology (motifs 1-4, Figure 6.1 & Table 5.1) (Westaway *et al.*, 1994a). It was suggested that these conserved motifs may be binding sites for as yet unidentified transcription factors. However, to date, specific binding to these sites has not been demonstrated, and few candidate factors have been identified from sequence analysis of these motifs. O'Neill *et al.* (2003) noted that motif 2 shares sequence homology with the human bZIP repressor protein, and motif 4 shares homology with conserved sequences in muscle-specific genes (Inoue *et al.*, 1997, Westaway *et al.*, 1994a). In sheep a T to C polymorphism within motif 1 (TCATTTT versus CCATTTT) has been identified (O'Neill & Cairns, Unpublished). In addition, motif 2 contains a species-specific sequence variation, where the ruminant PrP promoter contains a C within the motif (TTACGTAA), whilst the non-ruminant promoter contains a T at the same position (TTATGTAA). Similarly, the PrP promoter contains a species-specific C to T variation in motif 3, with the rat promoter containing a C within the motif (TAAAGATGACTTTTA), whilst the other mammalian promoters contain a T at the same position (TAAAGATGATTTTTA). These variations/ polymorphisms provide complex targets for the investigation of an association between the role of transcription factors and regulation of the PrP gene. Therefore this study focused on the characterisation of the conserved motifs in the ovine PrP promoter with the specific aims of identifying candidate transcription factors and analysing the binding activity of nuclear proteins to oligonucleotide probes containing the motif sequences.

As yet it is not known whether the motifs are actually occupied by DNA binding proteins. Potential binding of nuclear protein factors to these motifs was assessed by gel shift assays carried out with nuclear extracts from the ovine cell lines available at the NPU (Section 2.5). For a description of the gel shift assay technique see section 5.1. Finally, work was carried out to identify the proteins which bind to

the motifs using antibodies specific for the candidate proteins in gel super-shift assays. These assays are similar to the gel shift assay, however, the addition of an antibody specific for a protein present in the binding assay results in a further shift of the DNA-protein complex (super-shift). It is proposed that this study will provide insights into the potential role of the PrP promoter motifs in the regulation of PrP gene expression. In order to assess their relevance to disease control, the results of this work were analysed with respect to the cell lines from scrapie susceptible and resistant sheep that are available at NPU.

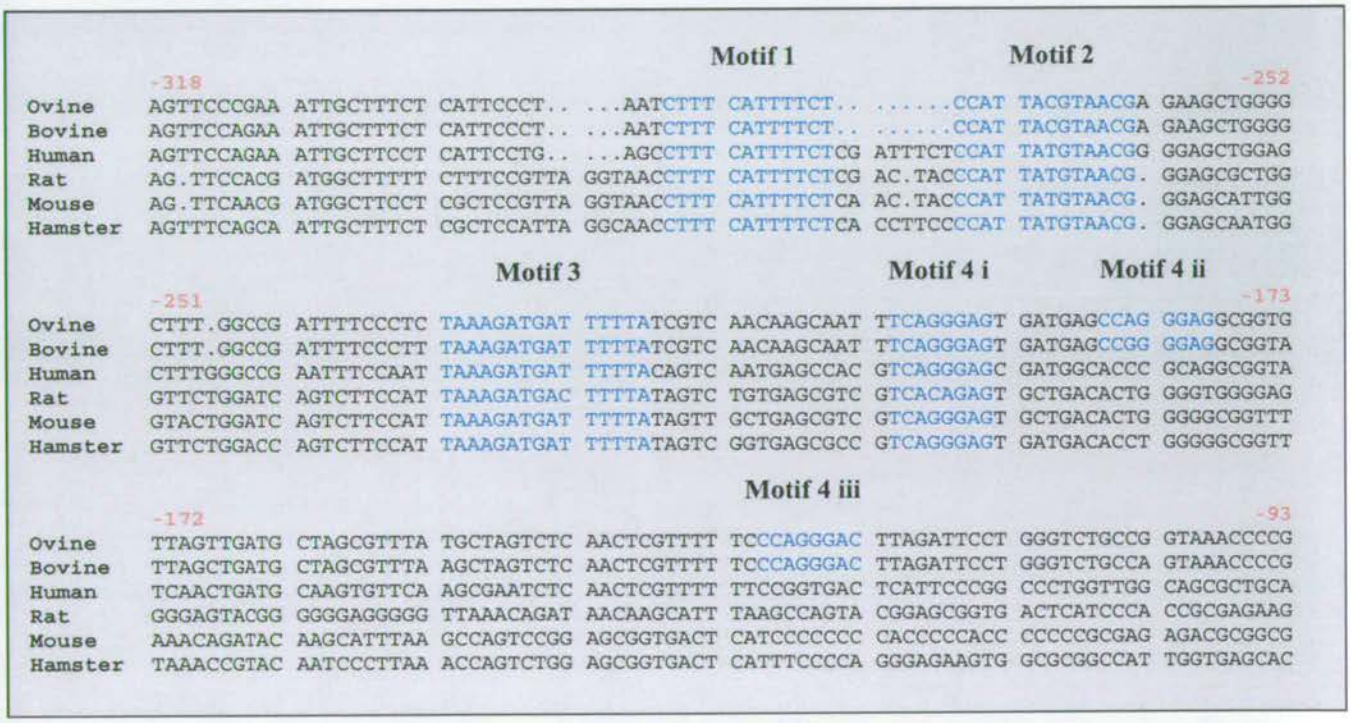


Figure 6.1 *Mammalian PrP promoter motifs*

Motifs 1-4 (indicated in blue text) are conserved sequence elements found in ovine, murine, hamster, cattle and human PrP gene promoters, as identified by Westaway *et al.* (1994a) which may form binding sites for as yet unidentified transcription factors. Numbering is based on the use of the 5' transcription start site as experimentally defined by Westaway *et al.* (1994a) as +1. NB: Sequence alignments performed on GCG (Wisconsin) using local pile-up program, artificial gaps (indicated by dots) were introduced into the above sequences in order to line-up potential transcription start sites.

6.2 Results

Complimentary single stranded oligonucleotides (approximately 20 bp in length) containing each of the four conserved motif sequences, along with the motif 4 degenerate repeat sequences (motif 4 (i), (ii) & (iii), were annealed to form double stranded oligonucleotides (Section 2.4.1) and radiolabeled with ^{32}P (Section 2.4.2) (Table 6.1). These oligonucleotides were incubated in binding assays (Section 2.4.6) with nuclear extracts (Section 2.4.3) prepared from the ovine cell cultures (IS120Cer, IS120Liv, sA80BR & pA80BR) and the murine neuroblastoma cell line (N2a) (Section 2.5). The amount of nuclear extract added in each assay was between 1-2 µl (10-20 µg of total nuclear protein per binding assay). Care was taken to ensure that the same batch of nuclear extract was used for each reaction in a single binding assay and comparisons of mutated and wild type DNA sequences were made within the same batch of nuclear extract. All of the assays in this chapter were repeated on at least three occasions with similar results and always using different batches of nuclear extracts, unless stated otherwise. The DNA sequences of the oligonucleotides used for each of the binding assays can be seen in table 2.3, the sequences used to flank the motif sequence were the same as those observed in the actual ovine PrP promoter sequence.

Name	Abbreviation	DNA binding motif	Location
Wild type motif 1	M1T	5'CTTTCATTTTCTCCA'3	-286
Variant motif 1	M1C	5'CTTCCATTTTCTCCA'3	-286
Ruminant motif 2	M2C	5'TTACGTAACG'3	-271
Non-ruminant motif 2	M2T	5'TTATGTAACG'3	-271
Motif 3	M3	5'TAAAGATGATTTTTA'3	-232
Motif 4 (i)	M4i	5'TCAGGGAG'3	-201
Motif 4 (ii)	M4ii	5'CCAGGGAGG'3	-186
Motif 4 (iii)	M4iii	5'CCAGGGAC'3	-130
Variant M1 + Ruminant M2	M1C/M2C	5'TAATCTTCCATTTTCTCCATTACG TAACGAGA'3	N/A
Wild type M1 + Non-ruminant M2	M1T/M2T	5'TAATCTTTCATTTTCTCCATTATG TAACGAGA'3	N/A
Variant M1 + Non-ruminant M2	M1C/M2T	5'TAATCTTCCATTTTCTCCATTATG TAACGAGA'3	N/A
Wild type M1+ Ruminant M2	M1T/M2C	5'TAATCTTTCATTTTCTCCATTACG TAACGAGA'3	N/A
Humanised Ovine M1 + M2	hovM1/M2	5'GAGCCTTCCATTTTCTCGATTTCT CCATTACGTAACGGGGG'3	N/A
Human M1 + M2	hM1/M2	5'GAGCCTTTCATTTTCTCGATTTCT CCATTATGTAACGGGG'3	N/A

Table 6.1 *PrP promoter motifs*

Details the sequences of the PrP promoter conserved motifs as described by Westaway *et al.* (1994a), which may form putative transcription factor binding sites. The location of each site is given relative to the 5' transcription start site as experimentally determined by Westaway *et al.* (1994a). Red text indicates the presence of a polymorphic or species specific base change.

6.2.1 Motif 1

6.2.1.1 Characterisation of ovine motif 1 binding

The T to C polymorphism within motif 1 (-286 bp, Table 6.1) was analysed for any affect on protein binding. A gel shift assay comparing the wild type motif 1 (M1T, top strand of double stranded motif 1 oligonucleotide = 5'TAATCTTTCATTTTCTCCAT'3) and variant motif 1 (M1C, 5'TAATCTTCCATTTTCTCCAT'3) oligonucleotides was performed with all of the ovine cell culture nuclear extracts (sA80BR, pA80BR, IS120Liv & IS120Cer). The results obtained with the sA80BR nuclear extract are shown in Figure 6.2.

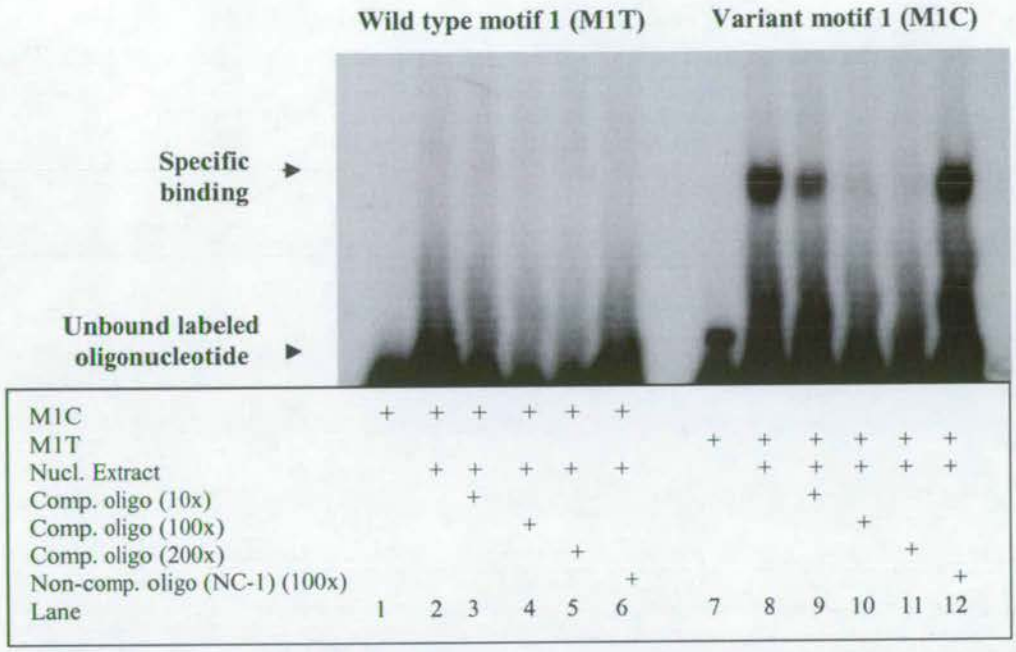


Figure 6.2 *Gel shift assay: motif 1 (M1C & M1T) oligonucleotides with sA80BR nuclear extract (NE)*

Gel shift assay indicating that the wild type motif 1 oligonucleotide (M1T) is not specifically bound by a transcription factor (lane 2), but the variant motif 1 oligonucleotide (M1C) is specifically bound by a transcription factor present in the sA80BR nuclear extract (lane 8). The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
sA80BR: nuclear extract from the sA80BR (scrapie susceptible) neuronal cell line
10X, 100X or 200X indicates the inclusion of a 10, 100 or 200 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

As can be seen, the M1C oligonucleotide bound strongly to a nuclear protein present in the sA80BR nuclear extract (Lane 8, Figure 6.2), and this binding was competed out by the addition of increasing concentrations (10, 100 & 200 fold molar excess) of unlabeled M1C oligonucleotide (Lanes 9, 10 & 11, Figure 6.2). In addition, competition with an unassociated non-competitor oligonucleotide (unlabeled NC-1, Table 2.3) had no effect on binding, indicating that the binding to

the M1C oligonucleotide was specific (Lane 12, Figure 6.2). However, the M1T oligonucleotide failed to show any specific binding in the ovine nuclear extract (Lane 2, Figure 6.2). The same result was also observed when this assay was repeated in the ovine pA80BR, IS120Liv and IS120Cer nuclear extracts (data not shown). In order to ensure that the observed results were not due to mistakes in the synthesis/ preparation of the oligonucleotides, both the M1C and M1T oligonucleotides were re-ordered and the gel shift assay shown in Figure 6.2 was repeated with these newly synthesised oligonucleotides with the same results.

6.2.1.2 *Variant motif 1 (M1C) oligonucleotide gel super-shift assay*

Search of transcription factor databases revealed the transcription factor Yin Yang 1 (YY1) (Shi, Lee & Galvin, 1997) to be a strong candidate for binding to the variant motif 1 sequence. The possibility that YY1 was the factor bound to the M1C oligonucleotide was further supported by comparisons with a purified murine AP-2 protein which has a molecular weight of 100 kDa (Lane 2, Figure 5.3). This comparison indicates that the factor bound to M1C (Lane 8, Figure 6.2) would have an approximate molecular weight of 50 kDa as it appears to have a mobility of approximately twice that of the purified murine AP-2 (Lane 2, Figure 5.3). To characterise the DNA/protein complex observed with the M1C oligonucleotide, an anti-YY1 antibody was employed in a gel super-shift assay. This antibody is reported to react with human, rat and mouse YY1 and is designed for use in gel super-shift assays (anti-YY1, Santa Cruz Biotechnology, USA). Assuming that the candidate factor had been correctly identified the antibody should bind to its epitope, and this binding may then cause one of two possible changes in the shift pattern. Firstly, the antibody may bind to the protein with no affect on protein-DNA binding, causing the mobility of the observed DNA-protein complex to change, a so-called super-shift. Alternatively, by binding to the protein the antibody may disrupt the DNA-protein complex and cause the DNA-protein complex to dissociate, consequently the initial shift will either be reduced or completely blocked (Bakalkin, Yakovleva & Terenius, 1997, Johansson, Hjortsberg & Thelander, 1998). Super-shift assays were performed with M1C oligonucleotide incubated with sA80BR or N2a nuclear extracts.

The anti-YY1 antibody was added after the radiolabeled M1C oligonucleotide and the samples were incubated at room temperature for 45 minutes (Figure 6.3). No super-shift of the DNA-protein complex was observed, instead the addition of the anti-YY1 antibody resulted in the dissociation of the initial complex (Lane 3, Figure 6.3 and compare with Lanes 8-12, Figure 6.2). This result suggested that a specific reaction had occurred between the M1C DNA-protein complex and the anti-YY1 antibody. The super-shift assay was repeated in nuclear extracts prepared from the murine N2a cells with the same result (data not shown). In addition, the assay was repeated with a shorter incubation time of 20 minutes, and also with the anti-YY1 antibody being added before the radiolabeled M1C oligonucleotide and on each occasion the same result was observed (data not shown). As an additional control, the ruminant motif 2 (M2C) oligonucleotide was incubated in a super-shift assay with the same anti-YY1 antibody, and no change in the M2C DNA-complex was observed, indicating that the reaction observed with the M1C oligonucleotide was specific (Lane 9, Figure 6.3). Finally the assay was also repeated using the anti-E4BP4 antibody, the results showed that this antibody had no effect on the M1C DNA complex indicating that the blocking reaction seen with the anti-YY1 antibody was specific and not caused by a component of the antibody solution (data not shown).

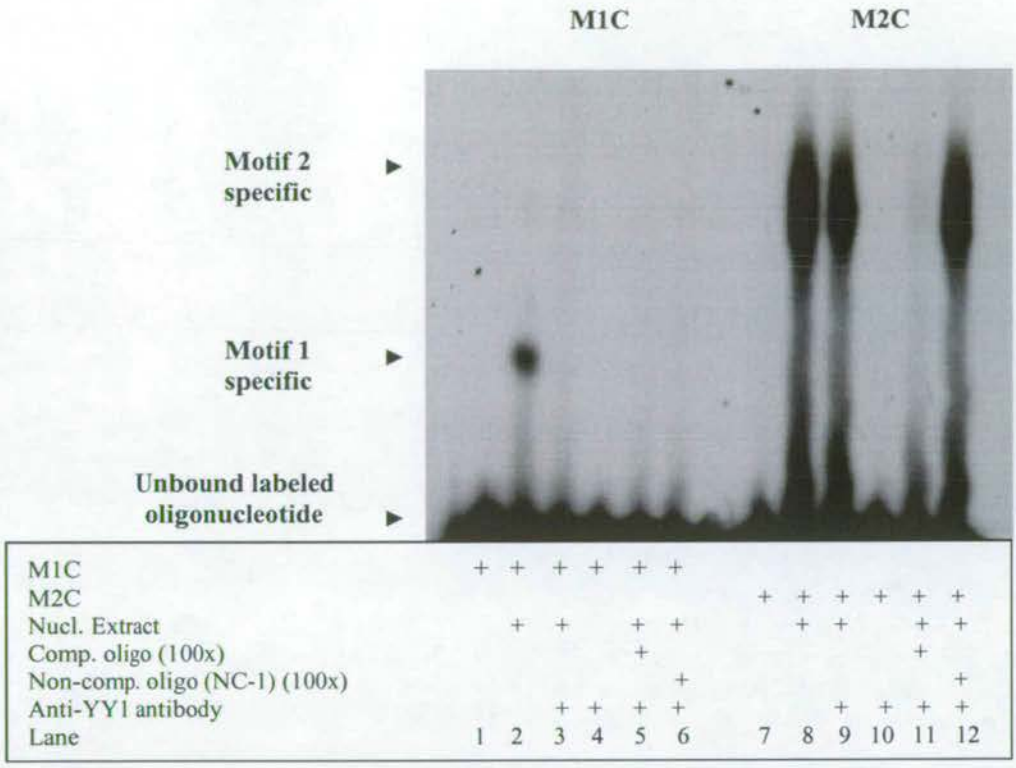


Figure 6.3 *Gel super-shift assay: variant motif 1 (M1C) and ruminant motif 2 (M2C) oligonucleotides with anti-YY1 antibody with pA80BR nuclear extract (NE)*

Gel super-shift assay indicating that the variant motif 1 oligonucleotide (M1C) is specifically bound by a transcription factor (lane 2) and this binding is ablated following the addition of an anti-YY1 (Yin Yang 1) antibody (lane 3). In addition the presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3. Antibody added was an anti-YY1 antibody (Santa Cruz, USA).

In order to further confirm this result, the M1C oligonucleotide was incubated with ovine sA80BR nuclear extract and increasing amounts of the anti-YY1 antibody (Figure 6.4). The amount of the shift complex (Lane 2, Figure 6.4) gradually decreased as more anti-YY1 antibody was added (Lanes 3-12, Figure 6.4). This

concentration dependent inhibition of the formation of a M1C DNA / protein complex provided further evidence that it is indeed YY1 bound to the M1C oligonucleotide.

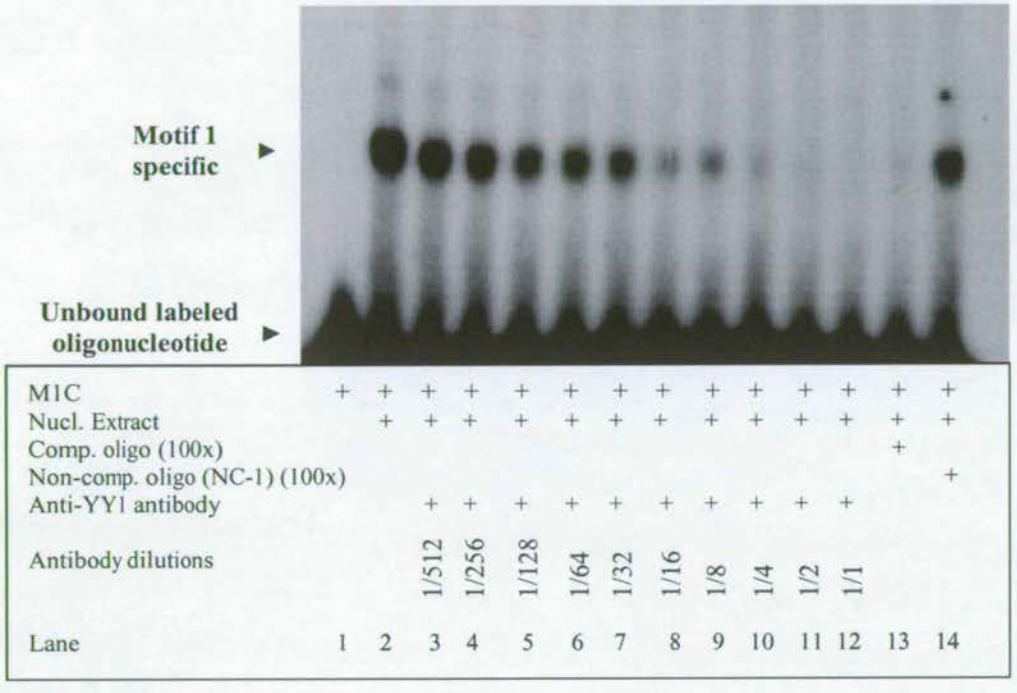


Figure 6.4 *Gel super-shift assay: variant motif 1 (M1C) oligonucleotide with increasing concentrations of anti-YY1 antibody with pA80BR nuclear extract (NE)*

Gel super- shift assay indicating that the variant motif 1 oligonucleotide (M1C) is specifically bound by the YY1 transcription factor (lane 2) and that this binding is inhibited by the addition of an anti-YY1 antibody in a concentration dependent manner. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3. Antibody added was an anti-YY1 antibody (Santa Cruz, USA).

6.2.2 Motif 2

6.2.2.1 Characterisation of motif 2 binding

A single base species difference of C to T (red text) is present in the non-ruminant PrP promoter, which changes motif 2 (-271 bp, Table 6.1) from TTACGTAA to TTATGTAA. Double stranded oligonucleotides containing both variations of motif 2 (ruminant motif 2 (M2C) & non-ruminant motif 2 (M2T), Tables 2.3 & 6.1) were produced (Section 2.4.1) and radiolabeled (Section 2.4.2) with ^{32}P. The affect of this species specific sequence difference on motif 2 binding was assessed by gel shift assays (Section 2.4.6) carried out with ovine cell and murine N2a cell nuclear extracts (only pA80BR and N2a results shown, Figure 6.5).

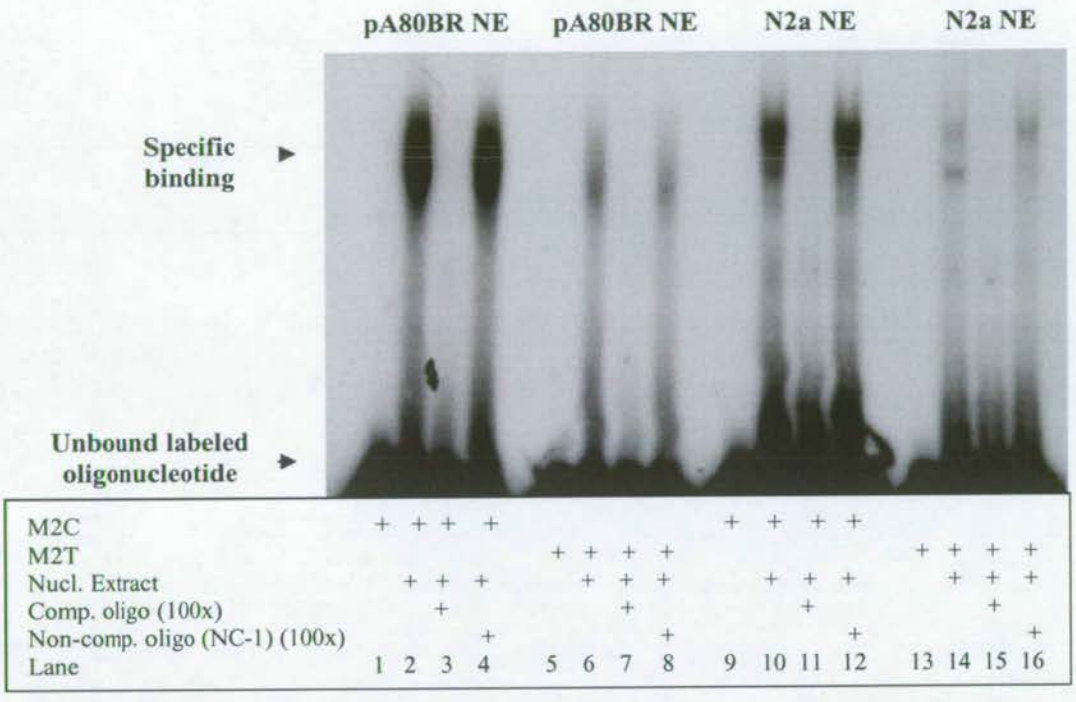


Figure 6.5 *Gel shift assay: ruminant (M2C) & non-ruminant motif 2 (M2T) oligonucleotides with pA80BR & N2a nuclear extracts (NEs)*

Gel shift assay indicating that the ruminant (M2C) & non-ruminant (M2T) oligonucleotides are specifically bound by a similar transcription factor (based on electrophoretic mobilities), however the factor appears to have a greater affinity for the M2C oligonucleotide, indicated by increased binding activity. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

The M2C oligonucleotide was shown to bind to a protein factor present in the ovine sA80BR, pA80BR, IS120Cer & IS120Liv cell nuclear extracts (only pA80BR shown) and also in the murine N2a cell nuclear extract (Lanes 2, 6 10 & 14, Figure 6.5). Binding was competed out by the addition of 100 fold molar excess unlabeled

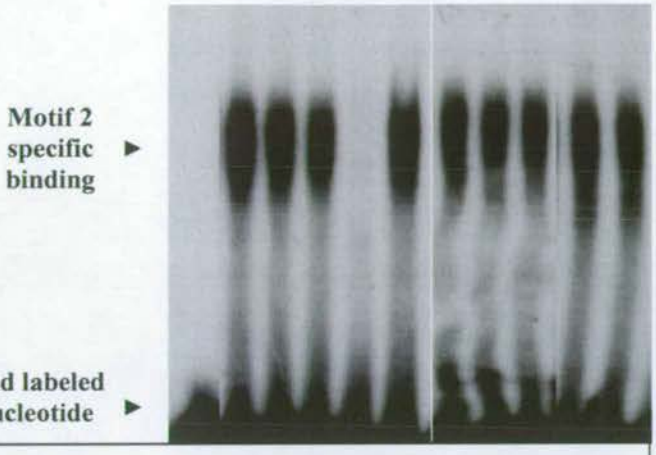
competitor oligonucleotide, M2C (Lanes 3, 7, 11 & 15, Figure 6.5). In contrast, binding was not affected by the addition of 100 fold molar excess non-competitor oligonucleotide (NC-1), indicating that the binding was specific (Lanes 4, 8, 12 & 16, Figure 6.5). The M2T oligonucleotide appears to bind to the same factor as the M2C oligonucleotide in the same nuclear extracts (Figure 6.5). However, the M2C oligonucleotide binds strongly to the protein factor (Lanes 2 & 10, Figure 6.5), whilst the M2T oligonucleotide binds to what appears to be the same factor, but with much lower affinity in the ovine cell culture and murine N2a cell nuclear extracts (Lanes 6 & 14, Figure 6.5).

The ruminant motif 2 but not the non-ruminant motif 2 contains a potential CpG island (TTACpGTAA). The effect of methylation at this site on protein binding was investigated by the use of a M2C oligonucleotide methylated at this position in gel shift assays (data not shown). No difference in binding affinity was observed between the methylated and unmethylated M2C oligonucleotides, indicating that methylation at this position does not effect protein binding (data not shown)

6.2.2.2 *Ruminant motif 2 oligonucleotide (M2C) gel super-shift assay*

A strong candidate for the factor binding to motif 2 is the bZIP transcription factor E4BP4, which is a dimeric PAR-like factor composed of two 52 kDa monomers (Cowell, 2002, Cowell *et al.*, 1992). This candidate was identified following analysis of the motif 2 sequence using the TFSEARCH and TRANSFAC databases. This is supported by comparisons of the protein bound to the M2C oligonucleotide with what are believed to be AP-2 (100 kDa) & SP-1 (106 kDa) bound to the dAP-2s & dAP-2c oligonucleotides, respectively (Lanes 2 & 8, respectively, Figure 5.5). These factors have similar mobilities to the protein bound to the M2C oligonucleotide (Lanes 2, 6, 10 & 14, Figure 6.5) indicating that this protein could be E4BP4 (molecular weight = ~100 kDa) (Cowell, 2002, Cowell *et al.*, 1992). More evidence for the binding of E4BP4 to motif 2 was provided by gel shift assays, which showed that the M2C oligonucleotide was bound strongly by a protein factor present in the cell culture nuclear extracts (Figure 6.5). In contrast, the M2T oligonucleotide was bound much more weakly by what appeared to be the same factor, based on comparisons of electrophoretic mobilities. E4BP4 recognises the

palindromic consensus motif of TTACGTAA, indicating that it would bind strongly to the M2C oligonucleotide (TTACGTAA), whilst binding of E4BP4 to the non-palindromic, M2T oligonucleotide (TTATGTAA) may be less likely (Cowell, 2002, Cowell *et al.*, 1992). The possibility that another PAR factor could be bound to motif 2 was ruled out as it has been shown that the PAR factors, unlike E4BP4, will bind with equal affinity to the TTATGTAA and TTACGTAA motifs, as such E4BP4 was considered to be the prime candidate for binding to motif 2 (Falvey, Marcacci & Schibler, 1996, Hunger, Brown & Cleary, 1994). In addition, the two PAR family bZIP factors, hepatic leukemia factor (HLF – two 33 kDa monomers) and the albumin gene promoter binding protein (DBP – single 34 kDa monomer) which recognise the same binding site as E4BP4 were ruled out as candidate factors for the binding to motif 2 on the basis of their molecular weights. An antibody is available which reacts with human, rat and mouse E4BP4 and is specifically designed for use in gel super-shift assays (anti-E4BP4, Santa Cruz Biotechnology, USA). Gel super-shift assays (Section 2.4.7) were performed with the M2C oligonucleotide incubated with pA80BR nuclear extract (Section 2.4.3). The super-shift assays (Section 2.4.7) were carried out by adding the antibody either before (Lanes 3-6, Figure 6.6), or after the addition of the radiolabeled oligonucleotide (Lanes 7-11, Figure 6.6). In addition, two different sets of incubation conditions were used, either 45 minutes at room temperature (Lanes 3-9, Figure 6.6) or overnight at +4 °C (Lanes 10-11, Figure 6.6). No super-shift of the original shift complex was observed with any of the variations tested in ovine pA80BR cell culture nuclear extracts (Lanes 3, 4, 7, 8, 9, 10 & 11, Figure 6.6).



M2C	+	+	+	+	+	+	+	+	+	+	+
Nucl. Extract		+	+	+	+	+	+	+	+	+	+
Comp. oligo (100x)					+						
Non-comp. oligo (NC-1) (100x)						+					
Anti-E4BP4 antibody			+	+	+	+	+	+	+	+	+
Lane	1	2	3	4	5	6	7	8	9	10	11

KEY: Conditions: Lanes 3-6, anti-E4BP4 added prior to radiolabeled oligo, incubated at room temperature (r/t) for 45 minutes; Lanes 7-9, anti-E4BP4 added after oligo, incubated at r/t for 45 minutes; Lanes 10-11, anti-E4BP4 added after oligo, incubated at +4 °C overnight. All samples were run on a single gel, which was reorganised using the draw facility on Microsoft™ Word.

Figure 6.6 *Gel super-shift assay: ruminant motif 2 (M2C) oligonucleotide, with anti-E4BP4 antibody with pA80BR nuclear extract (NE)*

Gel super-shift assay indicating that the ruminant (M2C) oligonucleotide is specifically bound by a transcription factor which is not recognized by an antibody specific for E4BP4. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3. Antibody used is an anti-E4BP4 antibody (Santa Cruz, USA).

The anti-E4BP4 antibody was further tested by carrying out a gel super-shift assay (Section 2.4.7), with murine N2a nuclear extracts (Section 2.4.3) with the M2C oligonucleotide. The reason for this is that although the anti-E4BP4 antibody has not

been shown to react with ovine E4BP4, it does react with the murine form of the protein (Santa Cruz Biotechnology, personal communication). It was therefore expected that the antibody would be able to react with the murine E4BP4, if present in the N2a nuclear extract (Figure 6.7).

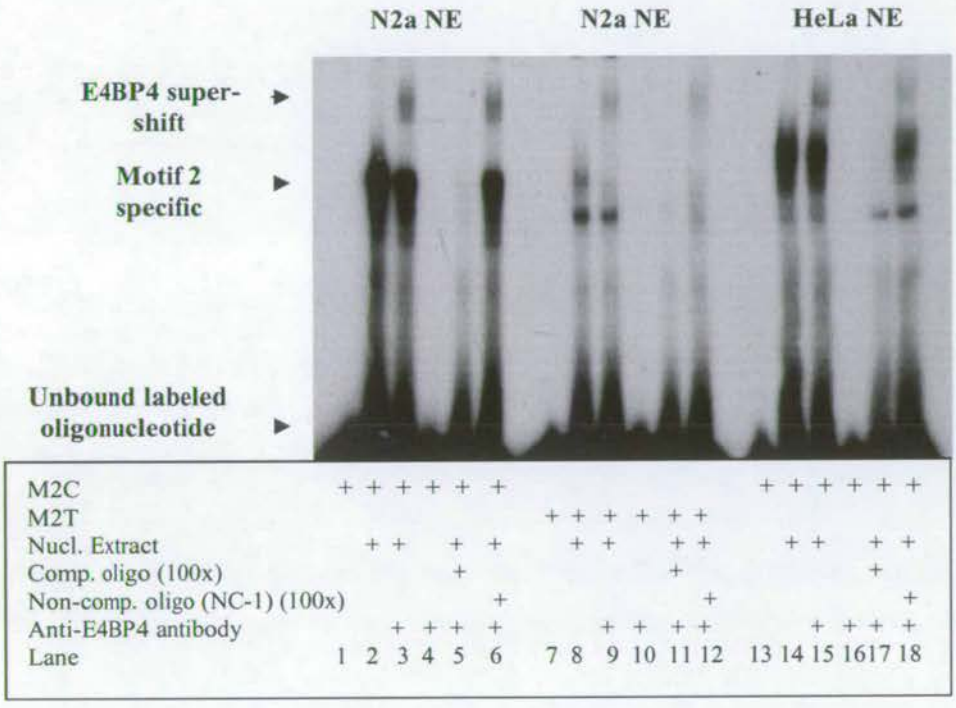


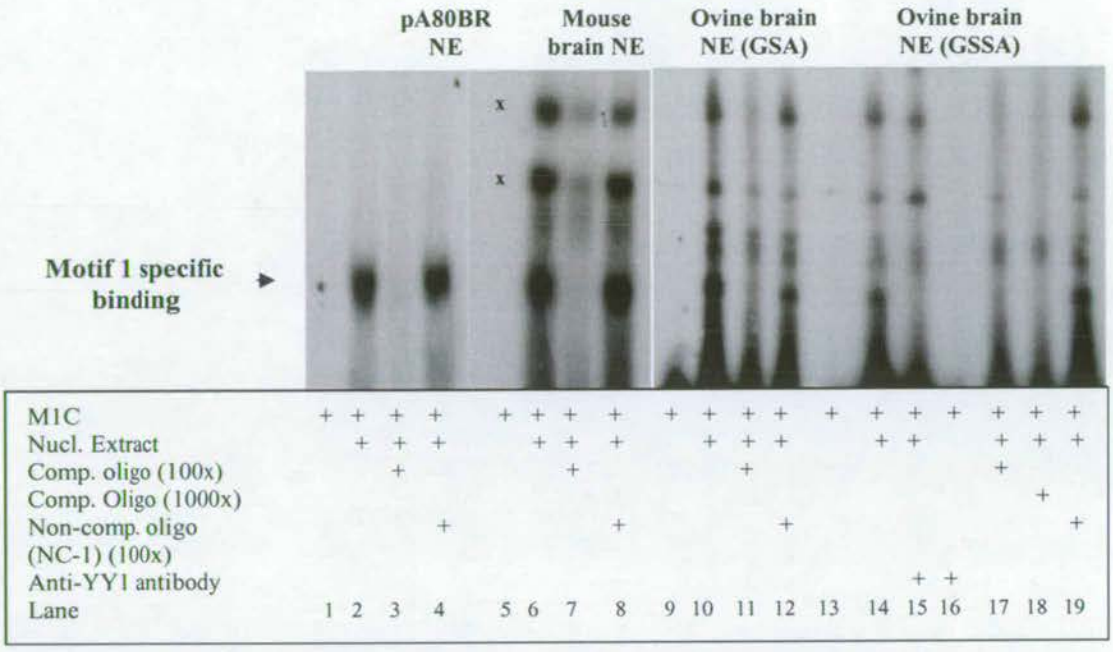
Figure 6.7 *Gel super-shift assay: motif 2 (M2C & M2T) oligonucleotides with anti-E4BP4 antibody with murine N2a and human HeLa cell nuclear extracts (NEs)*

Gel super-shift assay indicating that the transcription factor bound to the ruminant M2C (Murine N2a & Human HeLa nuclear extracts) & non-ruminant M2T (Murine N2a nuclear extract) oligonucleotides are specifically bound by a transcription factor which is recognized by an antibody specific for E4BP4. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
N2a: Murine neuroblastoma cell line nuclear extract
HeLa: Human cell line nuclear extract
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3. Antibody used is an anti-E4BP4 antibody (Santa Cruz, USA).

As can be seen from Figure 6.7, the anti-E4BP4 antibody reacted specifically with the original M2C DNA-protein complex formed with the murine N2a cell nuclear extract causing a super-shift to occur (Lane 3, Figure 6.7). In addition, no super-shift reaction was observed when the nuclear extract was omitted (Lane 4, Figure 6.7) indicating that the initial super-shift (Lane 3, Figure 6.7) was due to factors present in the nuclear extract and not a reaction between the M2C oligonucleotide and the antibody. Furthermore, the initial shift and the super-shift were both competed out by the addition of 100 fold molar excess unlabelled competitor oligonucleotide, M2C (Lane 5, Figure 6.7) but were not affected by the addition of unlabelled non-competitor oligonucleotide, NC-1 (Lane 6, Figure 6.7). The super-shift reaction (Section 2.4.7) was repeated with the M2T oligonucleotide with human HeLa cell line nuclear and murine N2a cell nuclear extracts and similar super-shifts were observed (Lanes 9 & 15, respectively, Figure 6.7).

6.2.3 Characterisation of binding to motifs 1 & 2 in nuclear extracts prepared from ovine and murine brain tissue

Binding to the M1C oligonucleotide was analysed with nuclear extracts prepared from fresh ovine (NPU Cheviot) & murine (C57BL) brain tissue (Section 2.4.4) (Figure 6.8).



NB: All shift assays were run on the same gel but were aligned using possible motif 1 binding factor (marked with arrowhead) using Microsoft ™ Word. M1C oligonucleotide additional specific complexes marked by '**x**'. GSA = gel shift assay, GSSA = gel super-shift assay.

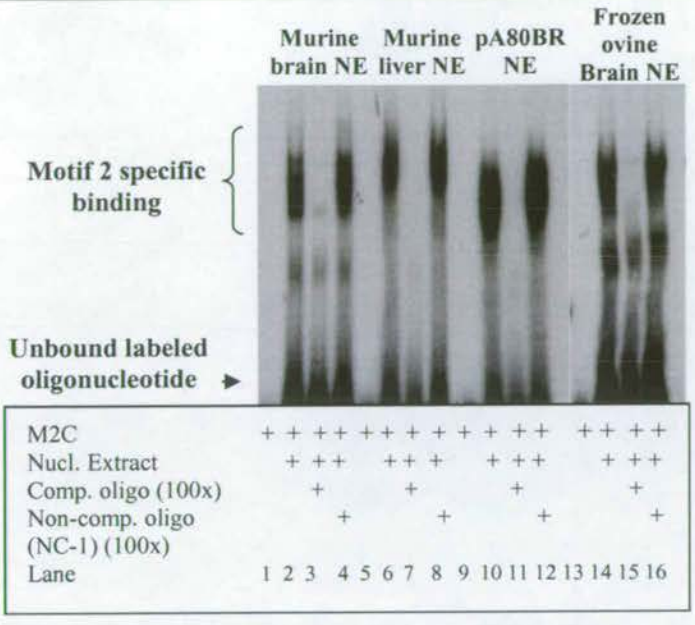
Figure 6.8 *Gel shift assay and gel super-shift assay: motif 1 (M1C) oligonucleotide with ovine & murine brain tissue derived nuclear extracts (NEs)*

Gel shift assay indicating that the variant motif 1 (M1C) oligonucleotide is specifically bound by three transcription factors, one of which is YY1. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
GSA: gel shift assay, GSSA: gel super-shift assay
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3. Antibody used is an anti-YY1 antibody (Santa Cruz, USA).

The electrophoretic mobility of the complexes observed in the binding assays carried out with nuclear extracts from the ovine and murine brain tissues (Figure 6.8) were consistent with those observed with the M1C oligonucleotide with the ovine

cell culture nuclear extracts (Lane 8, Figure 6.2). Specific binding was observed to the M1C oligonucleotide with murine brain nuclear extract (Lane 6, Figure 6.8) and the intensity of the binding detected was comparable to that observed with the same oligonucleotide with ovine pA80BR nuclear extract (Lane 2, Figure 6.8). Specific binding was also observed to the M1C oligonucleotide with the ovine brain nuclear extract (Lane 10, Figure 6.8). At least two other larger complexes were observed using the M1C oligonucleotide with the murine and ovine brain tissue derived nuclear extracts (Marked by 'x', Lanes 6 & 10, respectively, Figure 6.8) that were not observed in any of the other nuclear extracts analysed. The additional M1C oligonucleotide complexes were competed out by the addition of 100 fold molar excess unlabelled competitor oligonucleotide, M1C (Marked by 'x', Lanes 7 & 11, respectively, Figure 6.8). However, the complexes were not affected by the addition of 100 fold molar excess unlabelled non-competitor oligonucleotide, NC-1 (Marked by 'x', Lanes 8 & 12, respectively, Figure 6.8), indicating that they were specific. These additional complexes could represent an association between YY1, bound to the M1C oligonucleotide, and other transcription factors. In order to determine if the additional complexes contained YY1, a gel super-shift assay was performed with ovine brain nuclear extract using the anti-YY1 antibody (blocking assay), if the additional complexes were blocked then it would be reasonable to assume that they also contained the YY1 transcription factor (Lanes 13-19, Figure 6.8). However, only the lower band (similar mobility to YY1) was blocked by the anti-YY1 antibody (Lane 11, Figure 6.8), confirming that YY1 also binds to the M1C oligonucleotide in the ovine brain tissue nuclear extract. The additional complexes (Marked by 'x', Lane 10, Figure 6.8) are therefore likely to represent the binding of other, as yet unidentified, factors to the M1C oligonucleotide.

Binding to the M2C oligonucleotide was analysed with nuclear extracts prepared from murine (C57BL) brain and liver tissue and ovine (NPU Cheviot) brain tissue that had been flash frozen in liquid nitrogen and stored at –70 ° C for 2 weeks prior to the preparation of nuclear extracts (Section 2.4.4) (Figure 6.9).



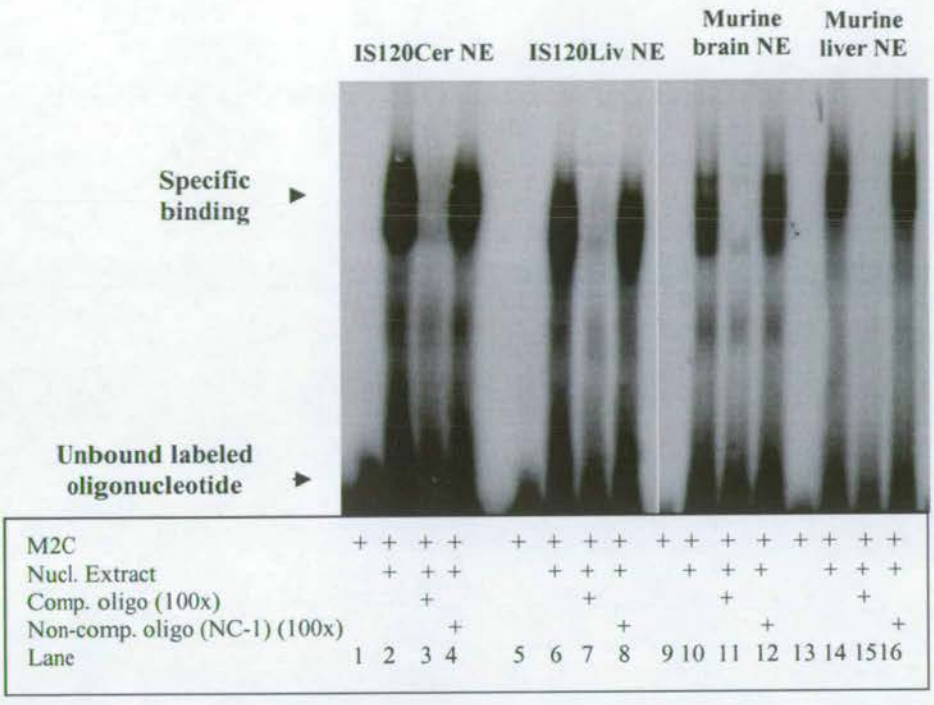
NB: Murine brain, liver & pA80BR shift assays from same gel. Ovine frozen brain tissue shift assay from separate gel

Figure 6.9 *Gel shift assay: motif 2 (M2C) oligonucleotide with ovine & murine tissue derived nuclear extracts (NEs)*

Gel shift assay indicating that the ruminant motif 2 (M2C) oligonucleotide is specifically bound by transcription factors with slightly differing electrophoretic mobilities in nuclear extracts derived from murine brain & liver and previously frozen ovine brain tissue. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

The complexes observed with nuclear extracts prepared from ovine and murine tissues (Figure 6.9) were consistent with those observed with the M2C oligonucleotide with the murine and ovine cell culture nuclear extracts (Figure 6.5). Specific binding was observed with the M2C oligonucleotide with the murine brain and murine liver tissue derived nuclear extracts (Lanes 2 & 6, Figure 6.9). In

addition, specific binding was observed to protein factors present in the nuclear extracts produced from frozen ovine brain tissue (Lane 14, Figure 6.9). The complex observed with the M2C oligonucleotide with mouse brain tissue nuclear extract, was slightly smaller than that seen with the mouse liver tissue nuclear extract (Lanes 2 & 6, respectively, Figure 6.9). A similar difference was also observed with the M2C oligonucleotide with the IS120Cer and IS120Liv nuclear extracts (Lanes 2 & 6, respectively, Figure 6.10). The complex observed with the IS120Cer nuclear extract (Lane 2, Figure 6.10) was slightly larger than that seen with the same oligonucleotide with the IS120Liv nuclear extract (Lane 6, Figure 6.10). These complexes are compared with the complexes from the murine brain tissue (Lane 10, Figure 6.10) and liver tissue nuclear extracts (Lane 14, Figure 6.10). The significance of these differences in mobilities is unclear, however, it is possible that they represent the binding of slightly different complexes (homo- and hetero-dimers) to the same motif in different tissue-types.



NB: Murine brain & liver NE gel shift assays from same gel, IS120Cer & IS120Liv NE gel shift assays from a single different gel

Figure 6.10 *Gel shift assay: comparison of ruminant motif 2 (M2C) oligonucleotide with liver & brain derived nuclear extracts (NEs)*

Gel shift assay indicating that the ruminant motif 2 (M2C) oligonucleotide is specifically bound by transcription factors with slightly differing electrophoretic mobilities in nuclear extracts derived from murine brain & liver tissue and from Icelandic sheep cell lines of neuronal and liver origin. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
IS120Cer: nuclear extract from the IS120Cer (cerebellum) cell line
IS120Liv: nuclear extract from the IS120Liv (liver) cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

6.2.4 Analysis of interactions between motif 1 and motif 2 binding factors

In the ovine PrP promoter the sequences specifying motifs 1 & 2 run almost directly into each other, and due to this close proximity it may be that the factors which bind to these motifs could interact in some way with one another. To test this hypothesis a series of double motif oligonucleotides were produced (Sections 2.4.1 & 2.4.2), which contained different combinations of motifs 1 and 2 and their variants (Tables 2.3 & 6.1). By using double motif oligonucleotide in gel shift assays it was expected to be possible to explore whether both factors could bind at the same time or whether double occupancy was not possible due to sterical hindrance or similar mechanisms. In the first case the mobility of that new complex would be distinct from that observed with either of the single motif oligonucleotides, in the second case similar retarded bands to those seen with the single motif oligonucleotides would be expected. Based on the results from the single oligonucleotide experiments, the strongest binding for both factors is predicted for the combination of variant motif 1 and ruminant motif 2 (M1C/M2C) generating the highest chance to observe the formation of an additional complex.

Additionally, oligonucleotides were used with a sequence insertion ('linker') between motifs 1 and 2, this arrangement resembles the human and mouse PrP promoter sequence. It was hypothesised that the 'linker' sequence between motifs 1 and 2 in these species could in some way influence the binding of YY1 and E4BP4 to these motifs. The first oligonucleotide contained the variant motif 1 (M1C) and the ruminant motif 2 (M2C), as observed in the ovine PrP promoter, separated by the 8 base linker (CGATTTCT) from the human PrP promoter (Humanised ovine M1 + M2 (hovM1/M2), Tables 2.3 & 6.1). A second oligonucleotide reflected the exact human PrP promoter sequence containing motifs 1 and 2 (Human M1 + M2 (hM1/M2), Tables 2.3 & 6.1) and was used as a control for the artificial hovM1/M2 oligonucleotide. The results are shown in Figure 6.11, only M1C/M2C, hovM1/M2 & hM1/M2 shown.

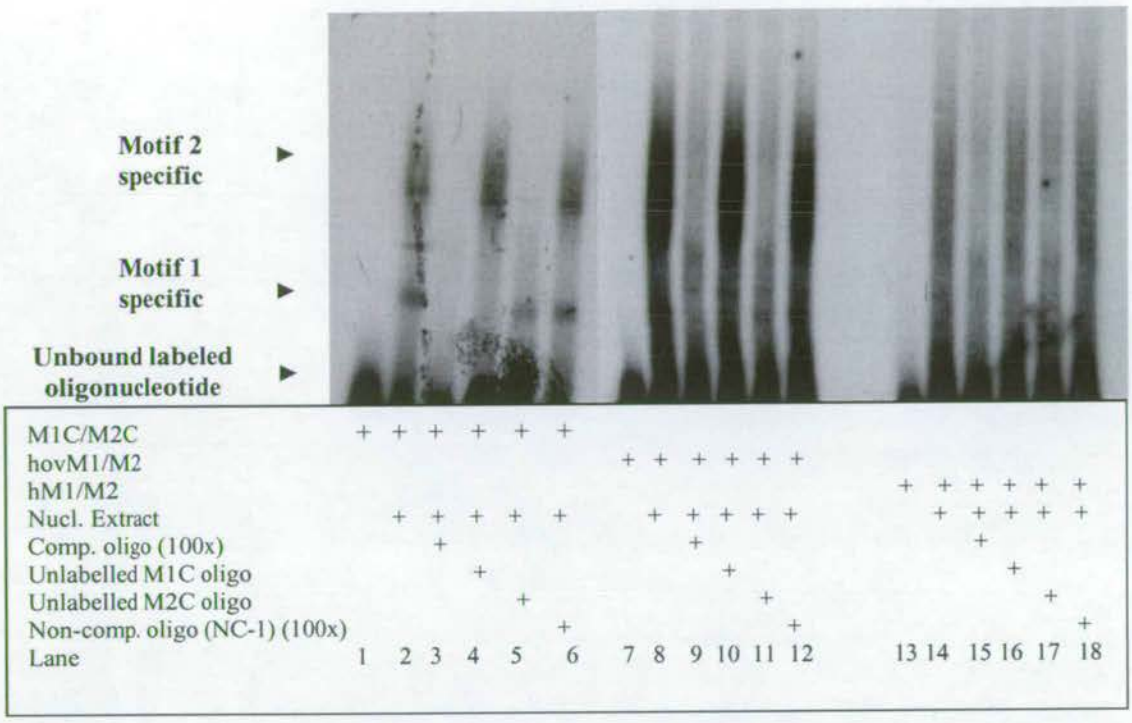


Figure 6.11 *Gel shift assay: motif 1 & motif 2 double oligonucleotides with pA80BR nuclear extract (NE)*

Gel shift assay indicating that a double motif oligonucleotide, containing both the variant motif 1 (M1C) and the ruminant motif 2 (M2C) sites is specifically bound only by the individual transcription factors and not by both factors at one time. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
100X indicates the inclusion of a 100 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

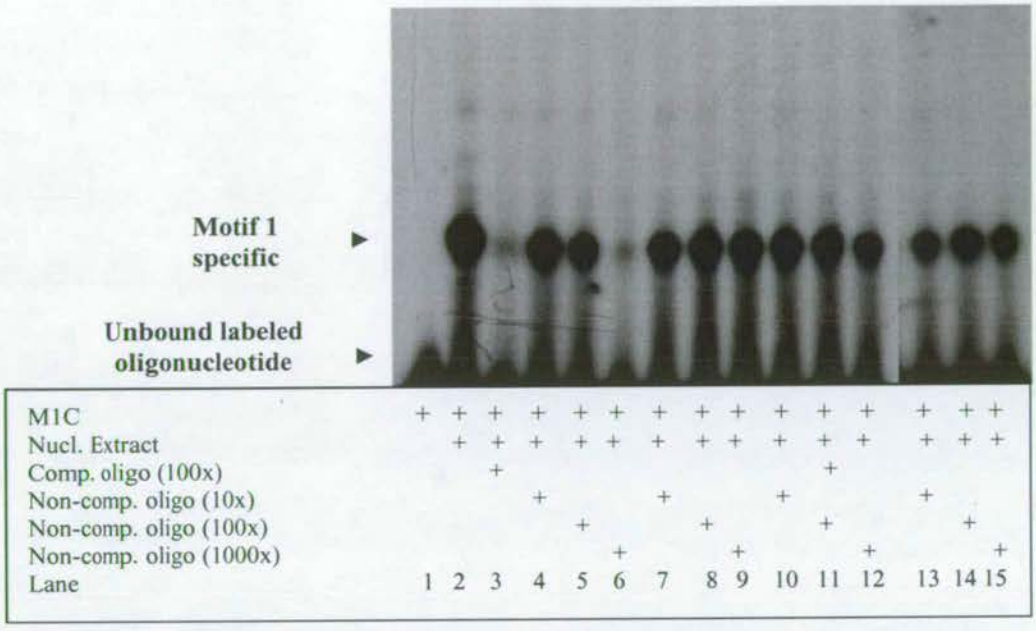
As can be seen from Figure 6.11, two individual retarded bands were observed with the M1C/M2C double oligonucleotide, which were consistent with the separate formation of a motif 1 complex or a motif 2 complex (Lane 2, Figure 6.11). The lower complex (motif 1 binding) was competed out by the addition of 100 fold molar excess unlabelled M1C oligonucleotide (Lane 4, Figure 6.11) and was not

affected by the addition of 100 fold molar excess unlabelled M2C oligonucleotide (Lane 5, Figure 6.11), indicating that the factor bound here was the same as that bound to the single M1C oligonucleotide (Lane 8, Figure 6.2). The upper complex (motif 2 binding) was not competed out by the addition of 100 fold molar excess unlabelled M1C oligonucleotide (Lane 4, Figure 6.11) and was competed out by the addition of 100 fold molar excess unlabelled M2C oligonucleotide (Lane 5, Figure 6.11), indicating that the factor bound was the same as that bound to the single M2C oligonucleotide (Lane 2, Figure 6.5).

At no time was an intermediate shift complex observed, indicating that the binding of these factors is mutually exclusive when presented with a close sequence motif arrangement (Lane 2, Figure 6.11). The binding to the artificial 8 base linker oligonucleotide (hovM1/M2, Lane 8, Figure 6.11) was similar to that observed with the M1C/M2C double oligonucleotide (Lane 2, Figure 6.11). Again it appeared that only one factor was able to bind at any one time to the double motif oligonucleotides tested (Lane 8, Figure 6.11). As expected no significant binding was observed to the hM1/M2 double oligonucleotide (Lane 14, Figure 6.11), a result consistent with that observed with the individual M1T (no binding, Lane 2, Figure 6.2) and the M2T oligonucleotides (weak binding, Lane 6, Figure 6.5) seen on the human PrP promoter. These experiments failed to identify any interaction between the factors bound to motifs 1 & 2, however, they indicated that the binding of these two factors to their respective binding motifs may be mutually exclusive.

Another method used to analyse interactions between the two binding factors in gel shift assays was to radiolabel only one of the two oligonucleotides in question (Section 2.4.2), in this case the M1C oligonucleotide. This labeled oligonucleotide was incubated in a binding assay (Section 2.4.6) with a nuclear extract and the resulting M1C DNA-protein complex was competed with an unlabeled M2C oligonucleotide. If an interaction occurred between the factors which bind to the two oligonucleotides (labeled M1C and unlabeled M2C), then this would be seen as a reduction in the original M1C DNA-complex. Interestingly, the results showed that the unlabeled M2T oligonucleotide competed out the M1C DNA-complex in a concentration dependent manner (Figure 6.12). The original M1C DNA-protein complex was almost completely competed out following the addition of 1000X

unlabelled M2C oligonucleotide (Lane 6, Figure 6.12). At this level the reduction in the amount of the original complex was similar to that observed following the addition of 100 fold molar excess unlabeled M1C competitor oligonucleotide (Lane 3, Figure 6.12). In addition, no reduction of the original variant motif 1 DNA-protein complex was observed with three different non-specific unlabeled competitor oligonucleotides (EGR-1, AP-1 & dAP-2c), indicating that the ruminant motif 2 competition may have been specific (EGR-1, Lanes 7-9; AP-1, Lanes 10-12; dAP-2c, Lanes 13-15, Figure 6.12).



KEY: Lanes 4-6 unlabeled M2C oligo; Lanes 7-9, unlabeled EGR-1 oligo; Lanes 10-12, unlabeled AP-1 oligo; Lanes 13-15, unlabeled dAP-2c oligo.

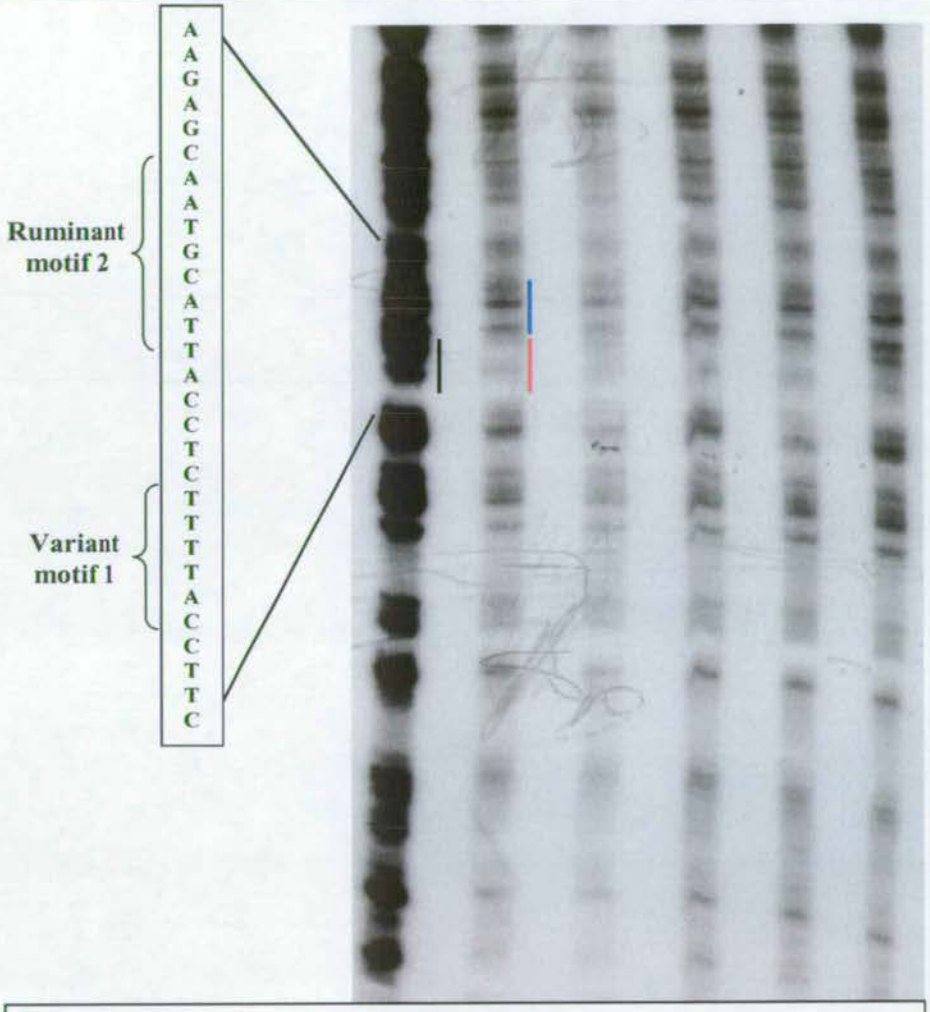
Figure 6.12 *Gel shift competition assay: labeled variant motif 1 (M1C) and unlabeled competitor & non-competitor oligonucleotides with pA80BR nuclear extract (NE)*

Gel shift assay indicating that a DNA protein complex formed by the variant motif 1 (M1C) oligonucleotide and the YY1 transcription factor is competed out by the addition of a 1000X excess of the ruminant motif 2 (M2C) oligonucletide, but is not affected by the same excess of the EGR-1, AP-1 or SP-1 oligonucleotides. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
10X, 100X or 1000X indicates the inclusion of a 10, 100 or 1000 fold molar excess of the appropriate unlabelled oligonucleotide. Sequences of the oligonucleotides used are detailed in Table 2.3.

6.2.5 DNase I footprinting of 0.5 kb ovine promoter region in ovine pA80BR nuclear extract

DNase I footprinting (Section 2.4.9) of the 0.5 kb Cheviot PrP gene promoter region was performed in order to supplement the results seen with the gel shift

assays. The footprinting method works on the basis that an interaction with a regulatory protein protects the DNA in the region of the binding motif from digestion by DNase I. The 0.5 kb promoter fragment was labeled at the 5' end with ^{32}P (Section 2.4.9). This fragment was incubated either with, or without ovine pA80BR nuclear extract (Section 2.4.3) and then digested with a sufficient quantity of DNase I to ensure that each molecule of the 0.5 kb Cheviot promoter DNA was cut at a single phosphodiester bond. The footprint product with no proteins attached to it produces a family of labeled fragments, which differ in size by one nucleotide appearing as a ladder of bands on the denaturing gel. In contrast, the footprint product with proteins attached to it produces an incomplete family of DNA fragments, with the absent fragments appearing as a footprint or protected area on the denaturing gel. The footprint was separated on a denaturing 4 % polyacrylamide gel (Section 2.3.4) and analysed for binding to the ovine variant motif 1 and ruminant motif 2 binding sites present on the 0.5 kb Cheviot promoter fragment. Additional footprints were carried out in the presence of unlabeled competing oligonucleotides (M1C & M2C), and a non-competing, unlabeled oligonucleotide (NC-1). Finally, the Cheviot 0.5 kb promoter fragment was sequenced by Sanger sequencing (Section 2.3) using a primer designed to bind specifically to the 5' end of the promoter (FP1, Table 2.2). This sequence was loaded on to the polyacrylamide gel next to the footprint sequence in order to allow identification of the motif sequences from the footprint analysis. The DNA sequence is represented in a diagrammatic form to the left of the DNase I footprint, which is shown in Figure 6.13.



0.5 kb promoter	+	+	+	+	+	+
Nucl. Extract (2 µl)		+		+	+	+
Nucl. Extract (4µl)			+			
Unlabelled M1C oligo (17x)				+		
Unlabelled M2C oligo (17x)					+	
Non-comp. oligo (NC-1) (17x)						+
Lane	1	2	3	4	5	6

KEY: Red line = variant motif 1 protected DNA footprint, Black line = variant motif 1 unprotected DNA footprint, Blue line = ruminant motif 2 unprotected DNA footprint.

Figure 6.13 *DNase I footprint: 0.5 kb ovine promoter fragment with pA80BR nuclear extract (NE)*

DNase I Footprinting assay of the Cheviot sheep promoter region containing motifs 1 & 2, indicating that the area containing motif 1 may be protected by bound nuclear proteins, whilst motif 2 may not be protected. The presence of each of the separate assay components is indicated by the + signs underneath each lane of the gel.
Comp. oligo: unlabelled competitor oligonucleotide, same sequence as the labelled probe. Non-comp. oligo: unlabelled non-competitor oligonucleotide, different sequence from the labelled probe.
Nucl. Extract: nuclear extract.
pA80BR: nuclear extract from the pA80BR (scrapie resistant) neuronal cell line
17X indicates the inclusion of a 17 fold molar excess of the appropriate unlabelled oligonucleotide.
Sequences of the oligonucleotides used are detailed in Table 2.3.

Following the addition of pA80BR nuclear extract the DNase I footprint of the 0.5 kb fragment indicated that there may be some protection in the region of the Cheviot promoter fragment containing the variant motif 1 (potential protection marked by red line, Lane 2, Figure 6.13). Furthermore, this section of DNA was clearly unprotected in the footprint performed without nuclear extract (Black line, Lane 1, Figure 6.13). In contrast, the area of DNA containing the ruminant motif 2 sequence did not appear to be protected following the addition of pA80BR nuclear extract (marked by blue line, lane 2, Figure 6.13). However, the potential protection of the variant motif 1 was not competed out by the addition of a 17 fold molar excess of unlabeled competitor oligonucleotides M1C (Lane 4, Figure 6.13) or M2C (Lane 5, Figure 6.13), nor was it affected by the addition of a 17 fold molar excess of an unlabeled non-competitor oligonucleotide, NC-1 (Lane 6, Figure 6.13). DNase I footprints of the Cheviot promoter fragment were performed on three separate occasions with the same result, however, time constraints did not allow further analysis.

6.2.6 Characterisation of motif 3 & motif 4 binding

Binding to the motif 3 (M3, -232 bp, Table 6.1) and motif 4 (i) (M4i, -201 bp, Table 6.1) oligonucleotides was assessed with the ovine cell culture nuclear extracts (sA80BR, pA80BR, IS120Cer and IS120Liv) by gel shift assay (Section 2.4.6). Only non-specific binding was observed with these oligonucleotides in all of the nuclear extracts tested (data not shown). The degenerate repeats of motif 4 (motif 4 (ii) and motif 4 (iii) (M4ii & M4iii, -186 & -130 bp, respectively, Tables 6.1 & 2.3) were analysed, and no specific binding was observed with any of the nuclear extracts tested (data not shown).

6.3 Discussion

During this study the PrP gene promoter conserved motifs 1 and 2 have been characterised in a variety of cell culture and tissue-derived nuclear extracts using a series of gel shift, gel super-shift, and DNase I footprinting techniques. The results of this study have shown that of the four PrP promoter motifs only two, motifs 1 and 2

appear to be bound by transcription factors under the assay conditions used in this study.

6.3.1 Motif 1

These experiments have shown that the wild type motif 1 sequence (M1T, CTTTCATTTTCT) as published for human, mouse and Suffolk sheep PrP promoters is not capable of forming a nuclear protein complex under the conditions used in the gel shift assays. However, a single base pair change from T to C, which has been found as a polymorphism in the ovine motif 1 sequence appears to have a dramatic effect on protein binding. The variant motif 1 (M1C, CTTCCATTTTCT) oligonucleotide shows strong, consistent and highly specific binding by gel shift assays in nuclear extracts from all ovine cell culture (Figure 6.2).

Sequence analysis of the wild type and variant motif 1 binding sites using the TFSEARCH and TRANSFAC databases revealed that the variant motif 1 sequence (CCATTTT) presents a potential binding motif for the YY1 transcription factor. Further evidence for this was provided by a comparison of the M1C DNA-protein complex with that observed with a recombinant murine AP-2 protein of known molecular weight (Figure 5.3). This showed that the molecular weight of the M1C DNA-protein complex would be expected to be approximately 50 kDa, a result consistent with a factor of a similar size to YY1 binding to this motif. The binding motif for YY1 contains either an ACATNTT core or a CCATNTT core (Hyde-DeRuyscher, Jennings & Shenk, 1995, Shi *et al.*, 1997, Yant, Zhu, Millinoff *et al.*, 1995). The YY1 transcription factor is a zinc finger protein with a predicted molecular weight of 44 kDa, it is located on a number of mammalian gene promoters and plays an important role in transcriptional regulation (Shi *et al.*, 1997). In contrast, the wild type motif 1 sequence (TCATTTT) does not form a consensus binding site for YY1, due to the presence of a T at position 1 which deviates from the core binding sequence of A/CCATNTT (Shi *et al.*, 1997, Yant *et al.*, 1995). This observation is further supported by Houbaviy, Usheva, Shenk *et al.* (1996) who produced a co-crystal structure of YY1 bound to DNA, which highlighted the importance of the CCATNTT core motif for binding. Interestingly, the interaction between the YY1 transcription factor and its DNA binding site depends more on

bonds formed with the complementary DNA strand. Therefore, YY1 forms strong interactions with the complimentary bases in the YY1 binding site, i.e. GGTANAA instead of CCATNTT (Houbaviy *et al.*, 1996, Hyde-DeRuyscher *et al.*, 1995). In particular YY1 forms strong interactions with the GG dinucleotide on the complimentary DNA strand (Houbaviy *et al.*, 1996). Therefore, it is highly likely that changes to the GG dinucleotide, as seen in the wild type motif 1 (AG), would affect the affinity of the YY1 protein for its DNA binding motif. This may explain the observed differences in binding of the motif 1 oligonucleotides (M1T & M1C), and suggests that YY1 is a candidate binding factor for variant motif 1. Interestingly, a similar situation has been described within the human cystic fibrosis transmembrane conductance receptor (CFTR) gene promoter region, where a single base change of A to T produces a novel YY1 binding site (Romey, Pallares-Ruiz, Mange *et al.*, 2000). The presence of this polymorphism causes a significant increase in the expression of the CFTR protein, and the authors hypothesise that this may have a beneficial effect on patients with cystic fibrosis.

The gel super-shift assay performed with the anti-YY1 antibody failed to produce a super-shift reaction. However, a specific blocking of the original M1C DNA-protein complex was observed (Figures 6.3 & 6.4). Interestingly, a number of researchers have reported a similar blocking reaction with the same anti-YY1 antibody (Bakalkin *et al.*, 1997, Johansson *et al.*, 1998). In contrast, Dyer & Rosenberg (2001) noted a definite super-shift reaction with the same antibody in their gel super-shift assays.

The YY1 transcription factor has defined roles in transcriptional repression and activation, it can also act as a transcriptional Inr element binding protein (Shi *et al.*, 1997, Smale, 1997). Intriguingly, it has been hypothesised that YY1 may act to repress the transcription of certain genes by causing the displacement of other regulatory factors, and YY1 sites are commonly found close to, or overlapping other binding sites (Ericsson, Usheva & Edwards, 1999). YY1 may repress transcription by recruiting histone deacetylases to the promoter (Shi *et al.*, 1997). In addition, YY1 is able to control gene transcription by associating with other regulatory proteins present on distant areas of DNA, and it is thought that YY1 achieves this by bending the DNA backbone to bring itself into close proximity with other regulatory factors,

such as SP-1 (Shi *et al.*, 1997). The ovine PrP gene promoter region has been sequenced in a limited number of sheep at the NPU, and these results have so far failed to show any strong association between the motif 1 polymorphism that creates the YY1 binding site and altered scrapie susceptibility (Dr Gerry O'Neill, NPU, personal communication). From the presented experiments it is not possible to predict how the binding of YY1 affects the expression of the PrP gene, indeed it may act as either a repressor, or an activator of transcription. Further studies will therefore be required to elucidate the effect of YY1 binding on transcription of the ovine PrP gene.

6.3.2 Motif 2

The ovine PrP gene M2C oligonucleotide was bound by a factor present in the ovine and N2a cell culture nuclear extracts (Figure 6.5). A species-specific sequence difference exists within motif 2, where the ruminant sequence is TTACGTAA, whilst the non-ruminant sequence contains a C to T change creating the following sequence TTATGTAA. The ruminant (M2C) and non-ruminant (M2T) motif 2 oligonucleotides were assessed for their ability to bind the same factor (Figure 6.5). The M2C oligonucleotide binds strongly to a protein factor, whilst the M2T oligonucleotide binds to what appears from gel mobility, to be the same factor but with much lower affinity (Figure 6.5). Interestingly, the same oligonucleotides bind to what appears to be the same factor from gel mobility, with the murine N2a nuclear extracts and with similar binding affinities (Figure 6.5). Sequence analysis of the ruminant and non-ruminant motif 2 binding sites using the TFSEARCH and TRANSFAC databases revealed that the ruminant sequence (TTACGTAA) forms a consensus binding site (TTACGTAA) for the bZIP repressor protein (Cowell *et al.*, 1992, O'Neill *et al.*, 2003). This is a transcription factor, also known as NF-IL3, and as E4BP4 (Cowell, 2002, Cowell *et al.*, 1992). The expression of E4BP4 is upregulated by cellular levels of both, interleukin-3 (IL-3) and glucocorticoids (Cowell, 2002, Cowell *et al.*, 1992, Wallace, Wheeler & Young, 1997). E4BP4 shares homology with the proline alanine rich (PAR) family of transcription factors (Cowell, 2002, Cowell *et al.*, 1992). However, whilst E4BP4 contains a PAR factor-like DNA binding domain, it lacks the PAR region required for transcriptional

activation (Cowell, 2002, Cowell *et al.*, 1992). Therefore, E4BP4 is able to compete for the same binding site as the PAR factors, but unlike PAR family members, E4BP4 acts as a repressor of transcription (Cowell, 2002, Cowell *et al.*, 1992, Ikushima, Inukai, Inaba *et al.*, 1997, Ishida, Ueda, Ohkawa *et al.*, 2000). The mechanism by which E4BP4 represses transcription remains unclear (Cowell, 2002). However, it may act by either displacing other factors from the same DNA site, or by binding site occlusion, preventing other factors from binding to the same site, and this may be controlled in a concentration dependent manner (Ikushima *et al.*, 1997, Ishida *et al.*, 2000, Lai & Ting, 1999). Ikushima *et al.* (1997) proposed that competition between E4BP4 and PAR factors for the same binding site could act as a form of rheostat switch. Therefore, as the concentration of one factor increases, it is able to bind to the DNA site, however, if hormones or other external stimuli act to increase the levels of the opposing factor, then it will dislodge the bound factor and bind itself to the DNA. In this way gene expression can be carefully controlled by the relative concentrations of two transcription factors, one that functions as a repressor of transcription and one that activates transcription (Ikushima *et al.*, 1997). E4BP4 is a member of the bZIP family of proteins which are characterised by their DNA binding (leucine zipper or bZIP) and dimerisation (basic region or bZIP) domains (Landschulz *et al.*, 1988, McKnight, 1991). This factor is formed by two monomers (homo- or hetero-monomers) coming together through a leucine zipper domain to form the dimeric E4BP4 transcription factor which is then, and only then, able to bind to its specific DNA binding site (McKnight, 1991). Haas, Cantwell, Johnson *et al.* (1995) showed that PAR family factors may be able to interact with other bZIP factors (like C/EBP and CREB/ATF) forming hetero-dimers, which would then be able to bind to imperfect palindromic DNA binding sites. The consensus binding site for E4BP4 is the palindromic sequence (5'TTACGTAA'3 and 3'TTACGTAA5'), and this is very common in dimeric transcription factor binding sites, as each monomer will bind to one identical half-site (Cowell, 2002, McKnight, 1991). The ruminant motif 2 sequence is palindromic (5'TTACGTAA3' and 3'TTACGTAA'5), however, the non-ruminant motif 2 sequence is not a palindrome (5'TTATGTAA'3 and 3'TTACATAA'5). Therefore, the single base change in the motif 2 sequence between ruminants and non-ruminants could have serious consequences for the

binding of a homo-dimeric transcription factor, and this is reflected in the results observed with the respective binding assays (Figure 6.5). The palindromic M2C oligonucleotide binds strongly to the factor in the nuclear extracts (likely to be E4BP4), whilst the non-palindromic M2T oligonucleotide binds with much lower affinity to what appears to be the same factor (Figure 6.5). Interestingly, these results are entirely consistent with those observed by Fujii, Shimizu, Toda *et al.* (2000) which indicated that a similar bZIP factor in yeast (Pap1) binds specifically to residues on the opposite strand of the Pap1 DNA binding site (TTACGTAA). Fujii *et al.* (2000) showed that interactions with the G in the sequence TTACGTAA were important in DNA binding. These results indicated that the palindromic nature of this motif is important for DNA binding to occur at an optimum level (Fujii *et al.*, 2000). bZIP factors may therefore be expected to show lower affinity for the non-palindromic motif 2 site observed in the non-ruminant PrP gene promoters. An important chemical modification, which may be involved in the regulation of the level of chromasomal packaging at the sites of expressed genes is cytosine methylation at 5'CG'3 dinucleotides, known as CpG methylation (Jones & Wolffe, 1999, Kundu & Rao, 1999, Selker, 1990, Wolffe, 1998). Methylation of DNA binding motifs may affect gene expression by interrupting the binding of proteins to these sites (Meehan, Lewis, McKay *et al.*, 1989, Tate & Bird, 1993). CpG islands are normally methylated, however, islands of unmethylated CpG exist where the proportion of 5'CG'3 dinucleotides is higher than average and these sites surround the promoter regions of certain genes (Kundu & Rao, 1999, Selker, 1990, Wolf & Migeon, 1985). The Suffolk sheep PrP gene (GenBank accession number = U67922) was mapped for the presence of potential CpG islands using the CpG island searcher program (Takai & Jones, 2003). The Suffolk PrP gene contains one CpG island which lies between bases 5279-6771 (a region which encompasses the Suffolk PrP gene promoter, exon I and intron I) and has a GC content of 59.4 %. Two regions within this CpG island have particularly high levels of 5'CG'3 dinucleotides, the first lies within the promoter region/exon I and spans 5567-5776 bp with 71 % of CG nucleotides. The second region lies within intron I and spans 6117-6316 bp with 74.5 % of CG nucleotides. The ruminant motif 2, but not the non-ruminant motif 2, contains a potential CpG island (TTACpGTAA). The methylation of cytosine at C-5

gives the cytosine residue a very similar structure to that of a thymidine residue. It would therefore be expected that the methylated oligonucleotide (5'TTA^{m}CGTAA'3) may have a similar effect on protein binding as the non-ruminant motif 2, which has a thymidine residue at the same position (5'TTATGTAA'3). As this was not the case, it is reasonable to assume that the corresponding adenine residue on the opposite strand of the non-ruminant motif 2 (3'AATACATT'5) has a greater affect on protein binding, further highlighting the importance of the palindromic nature of this motif for protein binding.

Gel super-shift assays of the M2C oligonucleotide with an anti-E4BP4 antibody with ovine nuclear extracts failed to produce a super-shift, or blocking reaction (Figure 6.6). However, when these super-shift assays were repeated, with murine N2a cell extracts a definite super-shift was observed (Figure 6.7). This reaction was also repeated, in a human HeLa nuclear extract, and an equally impressive super-shift reaction was observed (Figure 6.7). In addition, the M2T oligonucleotide was super-shifted with the N2a nuclear extract, although, the relative intensity of the original DNA-complex and the super-shifted complex was lower than that observed with the M2C oligonucleotide (Figure 6.7). The fact that the protein binding to motif 2 is specifically super-shifted by an anti-E4BP4 antibody with both murine and human cell culture nuclear extracts indicates that the factor, which binds to the M2C oligonucleotide with the ovine cell culture nuclear extracts may be E4BP4. The lack of a super-shift, or blocking reaction with this factor in the ovine cell extracts may be due to sequence changes in the ovine E4BP4 protein. These changes may result in a failure of the anti-E4BP4 antibody to recognise the ovine protein, and interestingly, the anti-E4BP4 antibody has to date, only been shown to react with the human, mouse and rat forms of E4BP4. These results indicate that, at least in mice and humans the factor which binds to motif 2 is the E4BP4 transcription factor and that this is very likely to be the factor which binds to motif 2 in sheep. However, the possibility that the factor bound to the M2C oligonucleotide in the ovine cell culture nuclear extracts could be another bZIP transcription factor, and possibly another PAR factor, cannot be ruled out. Although, further gel super-shift assays using antibodies specific for other bZIP factors could be used to confirm the identity of the bound nuclear protein.

The E4BP4 transcription factor has defined roles in the regulation of a number of cellular processes (Cowell, 2002). E4BP4 has been shown to play a pivotal role in the regulation of circadian rhythms, and this is extremely interesting from the point of view of TSEs, as PrP null transgenic mice with no functioning PrP gene have been shown to exhibit altered circadian rhythms (Cowell, 2002, Doi, Nakajima, Okano *et al.*, 2001, Mitsui, Yamaguchi, Matsuo *et al.*, 2001, Tobler *et al.*, 1996). In addition, Cagampang, Whatley, Mitchell *et al.* (1999) showed that PrP mRNA is itself regulated in a circadian manner, and furthermore, fatal familial insomnia is a TSE disease that results in patients suffering from interrupted sleep patterns (Gambetti, Petersen, Monari *et al.*, 1993). Therefore, the identification of a protein with defined roles in the regulation of circadian rhythms, which interacts specifically with the PrP gene promoter is potentially an important finding for the field of TSE research. The E4BP4 transcription factor has also been shown to have defined roles in the control of apoptosis and in signal transductance pathways (Furlow & Brown, 1999, Ikushima *et al.*, 1997, Kuribara, Kinoshita, Miyajima *et al.*, 1999, Nishimura & Tanaka, 2001, Wallace *et al.*, 1997, Yu, Chiang & Yen, 2002). Interestingly, apoptosis has been hypothesised as a possible mechanism of pathogenesis in prion diseases (Ferrer, 1999, Forloni *et al.*, 1996, Jamieson *et al.*, 2001, Jesionek-Kupnicka *et al.*, 1997, Kretzschmar *et al.*, 1997). There is some evidence for the involvement of PrP^c in signal transductance pathways (Chiarini *et al.*, 2002, Mouillet-Richard *et al.*, 2000, Spielhaupter & Schatzl, 2001).

As E4BP4 exhibits transcriptional repression by competing for the same binding site with members of the PAR family of bZIP transcription factors, this raises the possibility that the motif 2 site could be occupied by E4BP4 as a default therefore repressing PrP gene expression. This initial repression may be regulated by external stimuli, which lead to either an increase in the cellular concentration of factors competing for the same site or a reduction in the cellular concentration of E4BP4, therefore increasing the expression of the PrP gene. Therefore, elucidating the role that E4BP4 plays in the expression of the PrP gene is important if we are to further understand the effect, if any, that this factor has on scrapie susceptibility.

6.3.3 Motif 1 & 2 gel shift assays in tissue derived nuclear extracts

The gel shift assays of the M1C and M2C oligonucleotides were repeated with murine (C57BL) and ovine (NPU Cheviot) tissue derived nuclear extracts, and the results confirmed those seen with the cell culture nuclear extracts (Figures 6.8 & 6.9). The M2C oligonucleotide bound specifically to what appears to be the same factor (likely to be E4BP4), with mouse and sheep brain nuclear extracts (Figure 6.9). The M2C oligonucleotide was also able to successfully bind to a factor (likely to be E4BP4) in nuclear extracts prepared from ovine brain tissue, that had been previously flash frozen, and stored at –70 °C for two weeks prior to preparation (Lane 14, Figure 6.9). Interestingly, the mobilities of the complexes seen with the M2C oligonucleotide with the murine tissue extracts seemed to differ. The complex observed with the murine brain tissue nuclear extract was of approximately the same size (mobility) as the complex observed with the sheep brain derived pA80BR nuclear extract (Lanes 2 & 10, respectively, Figure 6.9). However, the complex seen with the murine liver tissue was slightly larger than that observed with the other nuclear extracts (Lane 6, Figure 6.9). In addition, the complexes observed with the M2C oligonucleotide with nuclear extracts prepared from the sheep primary cell cultures IS120Cer & IS120Liv derived from brain and liver respectively, showed differing mobilities (Figure 6.10). The complex observed with the IS120Liv nuclear extract was slightly smaller than that seen with the IS120Cer nuclear extract (Lanes 6 & 2, respectively, Figure 6.10). It is not clear why these complexes have different mobilities, however, it could be due to the binding of different monomer combinations to the oligonucleotides in the different tissue-types. For example, it may be that in brain tissue a homo-dimeric factor (likely to be E4BP4) binds to the M2C oligonucleotide, whilst in liver tissue a different combination, i.e. a heterodimeric factor binds to the same oligonucleotide, thus explaining the different mobilities. It could also be caused by the binding of completely different factors to this oligonucleotide in the two different tissue-types. In fact, E4BP4 has been shown to compete with other bZIP transcription factors for the same binding motif and this may be used to control the tissue specific expression of certain genes (Ikushima *et al.*, 1997). The binding of hetero-dimeric and/or different homo-dimeric transcription factors to motif 2 could be further analysed with different tissue derived nuclear

extracts by performing gel super-shift assays with the M2C oligonucleotide, utilising antibodies specific for a variety of bZIP factors.

The M1C oligonucleotide binds specifically to a nuclear protein factor (likely to be YY1) in the murine and ovine brain tissue nuclear extracts (Lane 6 & 10, Figure 6.8). Two additional specific complexes (slower mobility) were observed with the M1C oligonucleotide in the murine and ovine brain nuclear extracts. The possibility that these additional complexes were associated with YY1 was investigated with a gel super-shift assay using the anti-YY1 antibody (Lanes 13-19, Figure 6.8). The anti-YY1 antibody specifically blocked the lower complex, indicating that it was in fact YY1. In contrast, the two additional complexes were unaffected by the anti-YY1 antibody, indicating that they were not associated with YY1.

The ability to extract nuclear proteins from murine and ovine tissues, both fresh and frozen, and to obtain clear and effective shift results with these proteins is important if we are to further our understanding of the factors identified during this study. The use of fresh and frozen archive tissue in gel shift assays may enable us to analyse the role, and expression of these, and other factors in a variety of tissue types and at various stages of disease. In this way it may be possible to elucidate if there are differences in their expression in different cell- or tissue-types, and to analyse the role of these factors in the regulation of scrapie susceptibility.

6.3.4 Analysis of potential interactions between factors bound to ovine motifs 1 & 2

The close proximity of motifs 1 and 2 raised the possibility that the YY1 and E4BP4 transcription factors may interact in some way. In order to investigate this a series of double motif oligonucleotides were produced (Tables 2.3 & 6.1), and gel shift assays were performed with ovine pA80BR nuclear extracts (Figure 6.11). The results showed that in all of the combinations of the variant and wild type motifs tested, only one of the two factors appeared to be able to bind at any one time (Figure 6.11). In addition, at no time was a binding-intermediate observed, which may have indicated the binding of both factors to the same oligonucleotide at once (Figure 6.11). The non-ruminant PrP promoters contain a small gap between motifs 1 and 2

(7-8 bases), therefore, two double motif oligonucleotides were produced to analyse the effect of this gap on binding. The first oligonucleotide was that of the human PrP promoter motifs 1 and 2 (hM1/M2, Tables 2.3 & 6.1). A second oligonucleotide was produced which contained the variant motif 1 site and the ruminant motif 2 site, as observed in the ovine PrP promoter, separated by the 8 base gap from the human PrP promoter (hovM1/M2, Tables 2.3 & 6.1). The results showed that the gap appeared to have no effect on binding, and again it appeared that only one factor was able to bind at any one time (Figure 6.11). These results indicate that the binding of YY1 and E4BP4 to their respective DNA motifs may be mutually exclusive, and this may be caused by steric hindrance between the two factors, preventing them from both binding together.

Another method used to assess the interaction between the YY1 and E4BP4 transcription factors was to incubate a labeled oligonucleotide containing the binding site of one of the factors in a normal binding assay. The DNA-complex that forms was then titrated by the addition of an unlabeled oligonucleotide, which contained the binding site of the other factor. A radiolabeled M1C oligonucleotide was incubated with pA80BR nuclear extracts and the subsequent YY1 DNA-protein complex was titrated by the addition of an unlabeled M2C (E4BP4 binding) oligonucleotide (Figure 6.12). The results showed that the addition of 1000X unlabeled M2C (E4BP4 binding) oligonucleotide competed out the original YY1 DNA-protein complex, indicating that there may be an interaction between YY1 and E4BP4 (Figure 6.12). Furthermore, the addition of 1000X unlabeled non-specific oligonucleotides (EGR-1, AP-1 & dAP-2c) failed to titrate the original YY1 DNA-protein complex, indicating that the interaction between YY1 and E4BP4 was specific (EGR-1, Lanes 7-9; AP-1, Lanes 10-12; & dAP-2c, Lanes 13-15; Figure 6.12). The fact that the unlabeled dAP-2c oligonucleotide, which contains a consensus SP-1 binding site, failed to affect the YY1 DNA-protein complex indicated that YY1 and SP-1 may not interact with each other in this system. The transcription factor SP-1 has been shown to specifically interact with YY1 to regulate gene transcription in a number of other systems, and the presence of YY1 and SP-1 (dAP-2c) binding motifs on the ovine PrP promoter raised the possibility that these two factors could interact in some way (Lackner & Muzyczka, 2002, Lee

et al., 1993, Seto *et al.*, 1993, Vassias, Hazan, Michel *et al.*, 1998). However, the results in this study indicate that a reaction between SP-1 and YY1 may not occur with the single motif oligonucleotides used in this study, although as the two binding sites are over 200 bases apart, any interaction between YY1 and SP-1 may involve DNA bending, which may not have been accurately replicated in this study. Interestingly, YY1 and E4BP4 binding sites are also found adjacent to each other on the p6 promoter of the parvovirus B19, indicating that the positioning of these sites may have some biological significance (Raab, Bauer, Gigler *et al.*, 2001). In addition, Zhou, Gedrich & Engel (1995) showed that transcriptional repression by YY1 may be mediated by interaction with the bZIP factors ATF/CREB, indicating that an interaction between YY1 and E4BP4, which is also a bZIP factor, may be possible. A hypothetical model for the interaction of YY1 and E4BP4 on the ovine PrP promoter is presented in Figure 6.14.

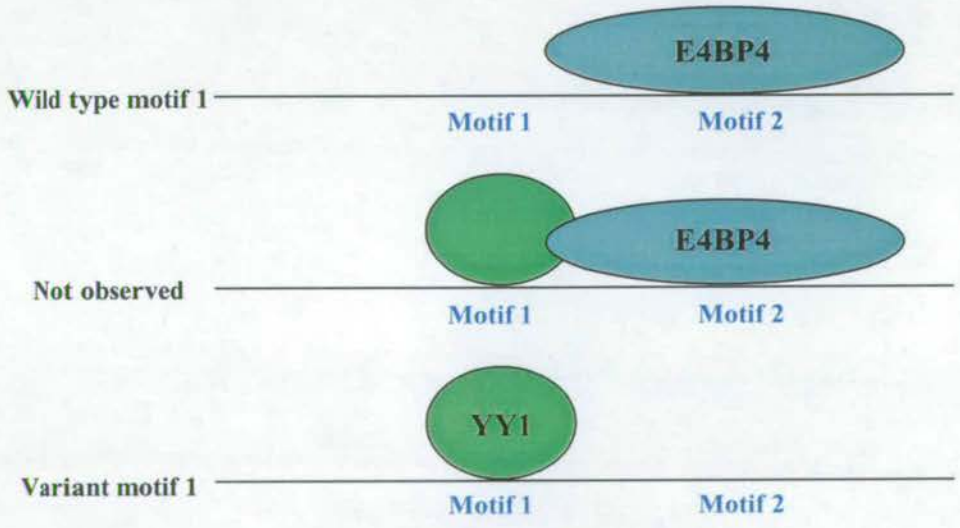


Figure 6.14 *Hypothesised interactions between YY1 and E4BP4 on the ovine PrP promoter*

Wild type motif 1 indicates the Suffolk sheep PrP promoter sequence, variant motif 1 indicates the Cheviot sheep PrP sequence. E4BP4 = the bZIP repressor protein (or E4BP4 transcription factor) also known as nuclear factor of interleukin 3 (NF-IL3). YY1 = the Yin Yang 1 transcription factor. Motif 1T = Suffolk sheep, rodent, bovine & human PrP promoter motif 1 sequence (TCATTTT), Motif 1C = Cheviot sheep polymorphic motif 1 sequence (CCATTTT), Motif 2C = bovine & ovine motif 2 sequence (TTACGTAA), Motif 2T = rodent & human motif 2 sequence (TTATGTAA).

As the binding of these two factors appears to be mutually exclusive, it is possible that one of the factors could act to dislodge the other. In the ovine PrP promoter, with the wild type motif 1 site, it is likely that only E4BP4 will bind, as no YY1 binding was observed to this motif (Figure 6.2). However, in an ovine PrP promoter that contains the variant motif 1 site it is likely that YY1 will bind. By doing so YY1 may prevent E4BP4 from binding to the motif 2 site, or it may act to dislodge E4BP4 from the motif 2 site due to steric hindrance (Figure 6.14). It is not clear what affect this would have on PrP gene expression, however, the dislodging of E4BP4, which is a repressor protein may act to increase the expression of PrP, therefore, YY1 could be acting as an activator of transcription in this situation. Exactly when a particular transcription factor with dual functions (like YY1) acts as an activator, or as a repressor, depends not only on its intrinsic features, concentration and on the presence of other transcription factors, but also on the structure, relative position, and orientation of the promoter itself (Roberts & Green, 1995, Tjian, 1995). Therefore, it is very difficult to predict how a single transcription factor will function on a given promoter, let alone how two individual factors will interact. Time constraints did not allow further analysis but a number of additional techniques are available to assess interactions between two proteins, these include the yeast two hybrid system, co-immunoprecipitation and the chromatin immunoprecipitation (ChIP) assay (Spencer, Sun, Li *et al.*, 2003). In addition a novel technique, the TranSignal™ TF-TF Interaction Array II (Panomics, USA) is available. This technique enables the researcher to determine how a particular transcription factor interacts with multiple other transcription factors, and could be used to further characterise the interaction between YY1 and E4BP4, and may provide a clearer picture of the role that these two factors play in PrP gene expression.

From the information available to date, it is unclear how the binding of the E4BP4 and YY1 transcription factors would affect PrP gene expression, or disease susceptibility. There are no obvious disease associations with the T to C polymorphism in the ovine PrP gene promoter motif 1 which creates the YY1 binding motif (Dr Gerry O'Neill, NPU, personal communication). Sequential deletion analysis of the motifs in a variety of mammalian PrP promoters failed to

reveal any significant effect on reporter gene expression (Baybutt & Manson, 1997, Funke-Kaiser *et al.*, 2001, Inoue *et al.*, 1997, Mahal *et al.*, 2001, Saeki *et al.*, 1996, Westaway *et al.*, 1994a), however, O'Neill *et al.* (2003) showed that deletion of the ovine motif 1 and truncation of motif 2 caused a slight reduction in expression of an ovine PrP promoter linked to a CAT reporter gene in sA80BR cells. However, the results of these deletion experiments should be carefully interpreted as a single deletion may remove more than one control element, in addition, two separate control elements may co-operate to produce a single response.

E4BP4 expression is controlled by cellular levels of IL-3, however, Lewicki, Tishon, Homann *et al.* (2003) showed that levels of IL-3 were not altered or were absent in TSE affected mouse brain. It should be remembered that E4BP4 is a repressing transcription factor, and by binding to the PrP promoter it would be expected to have a repressing affect on gene expression. Therefore, upregulation of E4BP4 would be unexpected in a TSE disease state, as PrP gene expression is crucial for the development of disease. It is possible that in the disease state factors other than IL-3, such as other cytokines, could be involved in the suppression of E4BP4 expression, or in the upregulation of factors that could compete for the same binding site with E4BP4. In fact, the expression of a number of cytokines has been shown to be increased in scrapie affected mouse brain, and the levels of certain transcription factors vary between the disease state and the healthy brain (Kim *et al.*, 1999, Park, Choi, Jin *et al.*, 2000). The expression of E4BP4 mRNA is upregulated by increased levels of cellular calcium (Nishimura & Tanaka, 2001), this is very interesting, as calcium regulation has been shown to be altered in TSEs, and PrP null mice show altered calcium homeostasis (Florio, Grimaldi, Scorziello *et al.*, 1996, Forloni, Angeretti, Chiesa *et al.*, 1993, Herms *et al.*, 2000, Herms *et al.*, 1997, Herms *et al.*, 2001, Kristensson, Feuerstein, Taraboulos *et al.*, 1993, Silei, Fabrizi, Venturini *et al.*, 1999, Wong, Qiu, Hyun *et al.*, 1996). Interestingly, E4BP4 mRNA is expressed at high levels in tissues, which are normally associated with low levels of PrP^c, such as the liver and lung, in contrast E4BP4 mRNA is expressed at much lower levels in brain, where PrP^c levels are normally high (Lai & Ting, 1999, Mitsui *et al.*, 2001). This observation suggests that E4BP4 may act to repress the expression of the PrP gene and that this repression could be carried out in a tissue-specific manner.

6.3.5 DNase I footprint analysis of PrP promoter motifs

Attempts were made to supplement the results seen with the gel shift assays using the DNase I footprinting technique (Galas & Schmitz, 1978). DNase I footprints of the 0.5 kb Cheviot PrP promoter fragment labeled with ^{32}P with ovine pA80BR nuclear extracts, revealed that the area of the DNA containing the variant motif 1 sequence may have been protected following the addition of pA80BR nuclear extract (Red line, Figure 6.13). The presence of a potential protected area of DNA at the same position as the variant motif 1 sequence on the Cheviot promoter may indicate that motif 1 was occupied by a nuclear factor (likely to be YY1). In contrast, the DNA sequence containing motif 2 did not appear to be protected following the addition of pA80BR nuclear extract (Blue line, Figure 6.13), a result consistent with YY1 binding to the Cheviot promoter at the variant motif 1, preventing the potential binding of E4BP4 to motif 2 (Figure 6.14). However, the potential variant motif 1 protected area was not inhibited by the addition of a 17 fold molar excess of unlabeled M1C and M2C competitor oligonucleotides (Figure 6.13), nor was it inhibited by the addition of a 17 fold molar excess of an unlabeled non-competitor oligonucleotide (Figure 6.13). Therefore, it is difficult to confirm the specificity of this protection. However, the addition of higher concentrations i.e 100 fold molar excess of the unlabeled competitor and non-competitor oligonucleotides may have resulted in the inhibition of this protection, and could be used to confirm if the observed potential protection was specific. In addition, the potential protection of variant motif 1 on the Cheviot promoter could be confirmed by repeating the DNase I footprint using the Suffolk promoter, which contains the wild type motif 1.

6.3.6 Motifs 3 & 4

No specific binding was observed in binding assays to the motif 3 (M3) and motif 4 (M4i, M4ii & M4iii) oligonucleotides with any of the ovine and murine nuclear extracts tested (data not shown). However, binding to these sites in abnormal cellular conditions cannot be ruled out, and it may be that these sites are, in fact, occupied, but only under specific cellular conditions, or in response to stimuli, or in tissue types not replicated/ assessed in these studies. Interestingly, motif 4 shares

homology with conserved sequences in muscle-specific genes, therefore, it may be prudent to analyse binding activity to motif 4 and its degenerate repeats in nuclear extracts derived from muscle tissue (Inoue *et al.*, 1997, Westaway *et al.*, 1994a).

6.3.7 Conclusions

In summary, the results observed in this study have characterised the conserved PrP gene promoter motifs 1 and 2. It was shown that ovine and murine cell lines contained nuclear proteins capable of binding specifically to an ovine motif 1 variant and to the ruminant motif 2. The use of antibodies specific for the candidate proteins YY1 and E4BP4 confirmed that YY1 is very likely to be the transcription factor, which binds to variant motif 1, whilst the factor binding to motif 2 is very likely to be E4BP4.

The binding studies also revealed a potential new mechanism of transcriptional regulation of PrP gene expression through the mutual exclusive binding of these two factors.

Chapter 7: Final discussion

7.1 Summary of results and discussion

The PrP gene is involved in the control of TSEs, as transgenic PrP null mice, which do not express endogenous PrP^c, exhibit resistance to infection (Bueler *et al.*, 1993, Manson *et al.*, 1999). It is hypothesised that the presence of even small differences in PrP^c expression levels, especially in the periphery, could lead to significant differences in the survival time of, or pathology of, individual animals (Hunter *et al.*, 1997). This is supported by scrapie infection experiments in PrP gene knockout mice, which showed a gene dosage effect, whereby homozygote PrP null mice were resistant to disease (ME7 scrapie strain) for at least 475 days, heterozygotes with one null allele and one functioning PrP allele showed mean incubation periods of 220 days and wild type mice with two normal PrP alleles showed incubation periods with a mean of 130 days (Manson *et al.*, 1994b). Amino acid variants of PrP^c particularly at codons 136, 154 & 171 have been linked to disease susceptibility in sheep. However, not all variation in sheep disease phenotype can be explained by polymorphic changes within the PrP gene open reading frame. It is hypothesised that other regions of the PrP gene involved in control of transcription and/or translation, for example the PrP gene promoter region and 3'UTR, may be involved in the modulation of disease pathogenesis. Therefore, unravelling the mechanisms that control the expression of the PrP gene is vital to our understanding of disease progression.

Alternative polyadenylation of the ovine PrP gene results in the production of two mRNA transcripts, a 2.1 kb and a 4.6 kb transcript, and in addition, a third 3.3 kb transcript may exist in peripheral tissues (Goldmann *et al.*, 1999, Horiuchi *et al.*, 1995, Hunter *et al.*, 1994c). This alternative polyadenylation may be involved in tissue-specific expression of the PrP^c protein as the 2.1 and 4.6 kb mRNA transcripts are found at different levels in different tissue-types (Goldmann *et al.*, 1999). However, the use of alternative polyadenylation does not fully explain certain observations made in gene expression experiments, as a PrP gene construct with a full length 3'UTR was expressed at lower levels than PrP constructs with shorter

3'UTRs (Goldmann *et al.*, 1999). More recently, Marshall (2000) confirmed that a sequence between 2000-2700 bp in the ovine PrP 3'UTR appeared to inhibit gene expression possibly via interactions with RNA binding proteins. The 2.1 kb transcript lacks this inhibitory region, and this may explain why a PrP construct with a full length 3'UTR was expressed at lower levels than those containing a shorter 3'UTR (Goldmann *et al.*, 1999, Marshall, 2000). In addition, this inhibition only occurred in ovine brain-derived cells, which encoded at least one copy of the ARQ allele. Therefore, it is possible that the expression of an RNA binding protein may either be limited to animals of specific PrP genotypes, or its expression may be higher in those genotypes (Marshall, 2000).

In order to further investigate the role of alternative polyadenylation on the expression of the PrP gene, a series of five PrP mini-gene constructs were produced (Chapter 4). The constructs differed only by their availability of polyadenylation signals located within the 3'UTR and were placed under the direct control of the 0.5 kb ovine PrP gene promoter. In addition, the ovine PrP ORF used in the construction of the mini-genes was tagged with the 3XFLAG™ epitope to enable the detection of recombinant PrP^c against a background of endogenous PrP^c normally expressed in these cells. The ovine PrP mini-gene constructs were then used to investigate the influence of alternative polyadenylation on PrP^c expression following the transient transfection of ovine and murine cell cultures.

Work carried out in the initial stages of this thesis resulted in the successful development of techniques for the extraction, immunoprecipitation and detection of endogenous PrP^c, from ovine and murine cell extracts and murine tissue (Chapter 3). Previous studies by Marshall (2000) had shown that endogenous PrP^c could be detected from approximately 2×10^7 N2a cells, in this study endogenous PrP^c was successfully detected from cell extracts prepared from 100-fold fewer cells (approximately 2×10^5 cells). Additional enhancements made to this system during this study, such as the use of a protein G sepharose matrix and a novel buffering system further improved the recovery of endogenous PrP^c from these cell cultures. The amount of PrP^c isolated from the cell cultures with the present system was comparable to that achieved by other researchers from cultured neuronal cells using similar methods (Lehmann & Harris, 1996, Parizek *et al.*, 2001, Winklhofer *et al.*,

2003). The techniques developed for the isolation of endogenous PrP^c during this study were used to extract recombinant PrP^c from ovine and murine cell lines which had been transiently transfected with the ovine PrP mini-gene constructs (Chapter 4). It was anticipated that the immunoprecipitation system developed during this study would be capable of successfully isolating recombinant PrP^c from the transiently transfected cell cultures. However, no recombinant PrP^c expression was detected from ovine and murine cell cultures transiently transfected with the ovine PrP mini-gene constructs. Sequencing of the ovine PrP mini-gene constructs confirmed their sequence integrity and highlighted the presence of the required sequence elements identified to date, including: 5' & 3' splice sites, a branch-point sequence and an ovine PrP gene intron region of approximately 700 bases. RT-PCR analysis of total RNA isolated from transiently transfected ovine cell cultures failed to detect any correctly spliced construct RNA, however the experiment was compromised by the presence in the reaction mixture of contaminating construct DNA due to the failure to treat the samples with DNase. In addition no control sample was included without the use of the reverse transcriptase reaction (a minus RT control). Due to the absence of important controls it remians unclear if the ovine PrP mini-gene construct RNAs were being correctly spliced or not, however incorrect splicing could also have been caused by the design of the constructs themselves, as they could be lacking sequence elements other than those identified in this study.

Following the failure of these mini-genes to be correctly processed and a lack of obvious sequence defects in the constructs it was decided not pursue the mini-gene constructs any further. Time constraints ruled out the production of a new series of constructs and it was entirely possible that even if a working series of mini-gene constructs were produced, the lack of specificity of the anti-FLAG™ antibodies, as determined in control experiments, may have interfered with, or nullified the experimental model. However, the problem of the anti-FLAG™ antibodies may have been overcome by instead using ^{35}S-methionine labeling to detect recombinant PrP^c expression. As such a decision was made to pursue the remaining aspects of the thesis, which involved analysing the role of the ovine PrP gene promoter in the regulation of PrP gene expression.

Gene expression is regulated by the binding of transcription factors to specific sequence elements within the promoter region (Mitchell & Tjian, 1989). These proteins aid in the formation of the basal transcription complex, in conjunction with RNA polymerase II (Mitchell & Tjian, 1989, Tjian, 1995). In addition, they can regulate the rate of transcription of a particular gene through specific interactions with other transcriptional elements (Mitchell & Tjian, 1989, Tjian, 1995). Before the work described in this thesis very few binding motifs for transcription factors had been identified in the ovine PrP promoter and only a single AP-2 motif (upstream AP-2), and an AP-2 motif cluster (downstream AP-2) in Suffolk sheep which is altered to a SP-1 motif following a C to G polymorphic change in Cheviot sheep, had been identified (O'Neill *et al.*, 2003) (Figure 5.1). The non-ruminant PrP promoters contain an AP-1 transcription factor binding motif, in addition, all of the mammalian promoters, with the exception of the Suffolk promoter, contain between two and three SP-1 binding motifs (Baybutt & Manson, 1997, Funke-Kaiser *et al.*, 2001, Inoue *et al.*, 1997, Mahal *et al.*, 2001, O'Neill *et al.*, 2003, Saeki *et al.*, 1996, Westaway *et al.*, 1994a). A number of polymorphic changes have been identified within the ovine PrP promoter including a polymorphic change of T to C and a single base polymorphism of C to A which is associated with the positive line (scrapie susceptible) of sheep in the NPU Cheviot flock.

Gel shift assays carried out in this thesis showed that the upstream ovine AP-2 motif (uAP-2) oligonucleotide was specifically bound by a protein factor present in the ovine cell culture nuclear extracts, and that this protein may be the AP-2 transcription factor (Chapter 5). In addition, the downstream ovine AP-2 motif cluster oligonucleotides (dAP-2s & dAP-2c), which represent the Suffolk and Cheviot sheep variants of this motif, respectively, were bound specifically by what appeared to be different nuclear proteins. Comparisons of the mobilities of these different factors suggested that AP-2 may bind to the Suffolk motif (dAP-2s), whilst SP-1 may bind to the Cheviot motif (dAP-2c). Suffolk and Cheviot sheep differ in genetic linkage with scrapie and this is not explained by polymorphic changes within the ovine PrP gene ORF (Hunter, 1997, Hunter *et al.*, 1994a). For example, Cheviot sheep in the NPU flock with the ARQ/ARQ genotype exhibit resistance to natural scrapie, whilst Suffolk sheep of the same genotype exhibit a high degree of

susceptibility to natural scrapie infection (Hunter, 1997, Hunter *et al.*, 1994a). One possible explanation of these observations is that the expression of PrP^c is higher in ARQ/ARQ Suffolks than in ARQ/ARQ Cheviots and that this is controlled by variation in the PrP gene promoter regions. A potential candidate for this variation is the polymorphic downstream AP-2 motif. From the data currently available it is not apparent how differential binding to this site in the Suffolk and Cheviot promoters would effect PrP gene expression. Deletion of SP-1 sites in the mouse, rat and cattle PrP promoters linked to a reporter gene resulted in a reduction in reporter expression, indicating that SP-1 may play an important role in the activation of PrP gene expression (Baybutt & Manson, 1997, Inoue *et al.*, 1997, Saeki *et al.*, 1996). Therefore, it is tempting to speculate that the putative binding of SP-1 to the ovine PrP gene promoter in Cheviot sheep may have a similar activating effect, thus resulting in the expression of the PrP gene. All of the mammalian PrP promoters analysed in this study, with the exception of the Suffolk sheep promoter, contain at least one SP-1 motif, therefore it is likely that the presence of a SP-1 motif represents the wild type situation of the PrP gene promoter. The putative binding of AP-2 to the Suffolk PrP promoter may therefore represent a variant situation, in that the putative binding of AP-2 to the downstream AP-2 motif in Suffolk sheep could result in the alternative activation of transcription, whereby the degree of activation exhibited by SP-1 and AP-2 could vary. O'Neill *et al.* (2003) showed that the upstream ovine AP-2 motif may activate PrP gene expression and this activating role may also apply to AP-2 bound to the downstream AP-2 motif cluster. Therefore, it is possible that differential binding of transcription factors to the downstream AP-2 motif cluster in Suffolk and Cheviot sheep of the ARQ/ARQ genotype could influence the rate of transcription of the ovine PrP gene. Differences in the level of PrP gene expression would be likely to affect the amount of PrP^c protein present, and may therefore affect the disease susceptibility of these two sheep breeds. These differences in expression could be exhibited in CNS tissues or in peripheral tissues involved in TSE pathogenesis, i.e. the lympho-reticular system. This is supported by studies which showed that the expression of the PrP gene in transgenic mice is directly related to disease susceptibility in a dose dependent manner (Manson *et al.*, 1994b). In addition, it is possible that even small differences in PrP^c expression levels,

especially in the periphery, could lead to significant differences in the survival time of, or pathology of, individual animals (Hunter *et al.*, 1997). Further studies using constructs containing the Cheviot and Suffolk PrP promoters linked to a reporter gene could be used to determine the influence of the C to G polymorphic change within the AP-2 cluster, following their transient transfection into ovine cell cultures.

During this study, the ovine PrP promoter was subjected to extensive sequence analysis and two potential initiator (Inr) elements were identified and mapped potential transcription start sites to within 1-3 bases of those identified previously (Westaway *et al.*, 1994b) (Chapter 5). These putative Inr elements are conserved to a certain extent in all of the mammalian species tested, although some sequence variations are present. Where the information was available these sites were found to be located within a few bases of previously identified transcription start sites (Funke-Kaiser *et al.*, 2001, Inoue *et al.*, 1997, Mahal *et al.*, 2001, Westaway *et al.*, 1994a, Westaway *et al.*, 1994b). Potential downstream promoter elements (DPEs) were also identified approximately +30 bases from the selected Inr elements in all of the mammalian PrP promoters analysed and the positioning of the Inr and DPEs may indicate the location of transcription start sites.

The initiation of transcription is the primary mechanism by which cells are able to regulate whether or not a particular gene is transcribed (Mitchell & Tjian, 1989, Tjian, 1995). Only the correct combination of protein factors will allow the initiation of a particular gene to be switched on, and the rate of this initiation can be regulated by interactions between the basal transcription complex and other transcription factors (Tjian, 1995). Therefore, the identification of potential Inr and DPEs, which play a critical role in the formation of the basal transcription complex, allows us to further understand the potential mechanisms which regulate the expression of the PrP gene. In addition, the presence of transcription factor binding motifs upstream of these elements may provide further means by which the rate of PrP gene transcription could be modified. For example, Inr elements have been shown to play a vital role in the formation of the basal transcription complex by allowing the binding of the TFIID complex to the promoter (Lo & Smale, 1996, Smale, 1997). The binding of TFIID to the Inr element may also occur in a cooperative manner with the AP-2 and SP-1 transcription factors (Laity *et al.*, 2001).

Therefore, the binding of either AP-2 or SP-1 to the downstream AP-2 motif cluster in the ovine PrP promoter may differentially influence the expression of the PrP gene by affecting the formation of the basal transcription complex. As such, the C to G polymorphic change within the downstream AP-2 motif could be related to disease susceptibility by control of the cellular PrP level.

Further sequence analysis of the ovine PrP promoter identified potential binding motifs for a number of transcription factors including HSE-1 & HSE-2, STAT family, AP-1, EGR-1 and GATA-1 (Chapter 5). No specific binding was observed to the HSE-1, HSE-2, AP-1, EGR-1, GATA-1 and STAT-C oligonucleotides. There was an indication of binding to the ovine STAT-A oligonucleotide, although the assay needs to be repeated for confirmation. The STAT family of transcription factors, are inducible cytoplasmic proteins, which can be activated to translocate to the nucleus by proinflammatory cytokines such as IFN-γ and IL-6 (Darnell, 1997, Ihle, 1996, Lackmann *et al.*, 1998, Leonard & O'Shea, 1998, Schindler & Strehlow, 2000, Takeda & Akira, 2000). Therefore, binding to the putative STAT motifs, which are conserved in the mammalian PrP promoters, requires further investigation and could be analysed with nuclear extracts prepared from cells which have been pre-treated with proinflammatory cytokines like IFN-γ. These experiments may reveal whether, or not the binding of STAT transcription factors to the PrP promoter is responsive to environmental changes, i.e. cytokine activation of cells. Interestingly, the expression of proinflammatory cytokines like IL-6 has been shown to be upregulated following scrapie infection and this could suggest a mechanism by which PrP gene expression could be altered in disease (Bacot, Lenz, Frazier-Jessen *et al.*, 2003, Kim *et al.*, 1999). For example, the upregulation of cytokines like IL-6 during a scrapie infection could potentially lead to the translocation of STAT transcription factors to the nucleus, where they may bind to the STAT binding motif on the PrP gene promoter. The binding of STAT proteins may act to increase the level of PrP gene expression, thus increasing the level of PrP^c and potentially increasing the spread of infectivity.

Binding to the four promoter motifs identified by Westaway *et al.* (1994a) was analysed and for the first time specific binding was observed to two of these motifs (motifs 1 & 2). No binding was detected to the wild type motif 1

oligonucleotide (M1T), however, specific binding was observed to a polymorphic variant of this motif (M1C). Further sequence analysis of this site revealed that only the variant motif 1 sequence formed a binding motif for the YY1 transcription factor and the specific binding of YY1 to this motif was confirmed by gel super-shift assay using an anti-YY1 antibody (Chapter 6). YY1 is a well-studied transcription factor which can act as either a repressor or an activator of transcription depending on its DNA sequence environment, i.e. interactions with other proteins (Lackner & Muzyczka, 2002, Lee *et al.*, 1993, Seto *et al.*, 1993, Shi *et al.*, 1997, Smale, 1997, Vassias *et al.*, 1998). YY1 can act as a transcriptional Inr element binding protein, and may act to repress the transcription of certain genes by displacing other transcription factors or by recruiting histone deacetylases to the promoter (Shi *et al.*, 1997, Smale, 1997). Interestingly, YY1 motifs are often found close to, or overlapping, other binding motifs (Ericsson *et al.*, 1999). Finally, YY1 is able to regulate gene transcription by associating with other regulatory proteins present on distant areas of DNA (Shi *et al.*, 1997). This is possible due to the ability of YY1 to bend the DNA backbone, bringing itself into close proximity with other regulatory factors, such as SP-1 (Seto *et al.*, 1993, Shi *et al.*, 1997). During the studies in this thesis no interaction was observed between YY1 bound to the M1C oligonucleotide and the dAP-2c oligonucleotide, which had been shown here to be bound by a nuclear protein with a similar electrophoretic mobility to SP-1. However, any potential interaction may have been dependent on DNA bending, which was unlikely to be replicated with the conditions and oligonucleotides used in this study.

Specific binding was observed to the ruminant motif 2 oligonucleotide (M2C) and binding affinity was reduced with the non-ruminant motif 2 oligonucleotide (M2T). The major candidate for binding to motif 2 was identified as the bZIP transcription factor E4BP4 and this was later confirmed by gel super-shift assay with an anti-E4BP4 antibody with murine and human cell culture nuclear extracts (Chapter 6). Therefore, it is likely that E4BP4 binds to the ovine motif 2, although the binding of another bZIP factor to the ovine motif 2 cannot be ruled out. E4BP4 is a repressing transcription factor, which is known to be involved in a variety of cellular processes including circadian rhythms, apoptosis and signal transduction, all of which are pathways important for PrP biology (Cowell, 2002).

Differential binding to the M2C oligonucleotide was revealed using ovine (NPU Cheviot) liver and brain tissue-derived nuclear extracts. Similar differences were also observed between murine brain and liver (C57BL) tissue-derived nuclear extracts. Interestingly, E4BP4 has been shown to compete with other bZIP transcription factors for the same binding motif and this can be used to control the tissue specific expression of certain genes (Ikushima *et al.*, 1997). Therefore, it is possible that motif 2 may be bound by one bZIP transcription factor, i.e. E4BP4, in one tissue-type and by another factor in a different tissue-type, thus suggesting a mechanism for the tissue-specific regulation of PrP gene expression. For example, previous studies have shown that E4BP4 mRNA is expressed at high levels in tissues that are normally associated with low levels of PrP^c, such as, the liver and lung (Lai & Ting, 1999, Mitsui *et al.*, 2001). In contrast, E4BP4 mRNA is expressed at much lower levels in brain, where the levels of PrP^c are normally much higher (Lai & Ting, 1999, Mitsui *et al.*, 2001). This may suggest that E4BP4 is able to act as a repressor of PrP gene expression, which could be tissue-specific and could be controlled by the binding of different bZIP factors to motif 2 on the PrP promoter. The tissue/ cell types analysed during this study have been limited to four ovine cell cultures and one murine cell culture. The cell cultures used were derived from either neuronal tissue (ovine sA80BR, pA80BR, & IS120Cer and murine N2a) or liver tissue (IS120Liv). The analysis of DNA binding to ovine promoter sequence elements, and especially to motif 2 with nuclear extracts prepared from other tissue/ cell types would therefore be highly desirable. The ability to successfully carry out gel shift assays with nuclear extracts prepared from fresh and frozen tissue samples provides the means by which binding to PrP promoter sequence elements could be analysed in a variety of tissue types including archive tissue samples and potentially disease state tissues.

The close proximity of motifs 1 and 2 on the PrP promoter raised the possibility of an interaction between the YY1 and E4BP4 transcription factors, suggesting a complex mechanism of transcriptional PrP regulation by these factors could exist. As the binding of these two factors appeared to be mutually exclusive, it is possible that one of the factors could dislodge the other, or prevent it from binding to the PrP promoter (Figure 7.1). In the ovine PrP promoter with the wild type motif 1 (sheep haplotype 1), it is likely that only E4BP4 will bind (Figure 7.1). However,

in an ovine PrP promoter that contained the variant motif 1 (sheep haplotype 2) it is likely that YY1 will bind, dislodging E4BP4 from motif 2 due to steric hindrance (Figure 7.1). Finally, the rodent/human haplotype of the wild type motif 1 and the non-ruminant motif 2 (Figure 7.1) is likely to be bound only by E4BP4. However, the binding of E4BP4 to the rodent/human PrP promoter will be with a lower affinity than that observed with the sheep haplotype 1 (Strong binding affinity indicated by dark blue E4BP4, weaker binding affinity indicated by light blue E4BP4, Figure 7.1). Therefore, differential binding to motifs 1 and 2 as observed with the three haplotypes presented in Figure 7.1, could potentially lead to species-specific differences in PrP gene expression. However, this idea is based on the limited number of mammalian promoter sequences tested to date, and in order to ascertain if more haplotypes exist, further promoter sequence analysis will be required.

As E4BP4 is a repressing transcription factor its binding to the PrP promoter would be expected to have a repressing affect on gene expression. Therefore, by dislodging E4BP4, the YY1 transcription factor could be functioning as an activator of transcription.

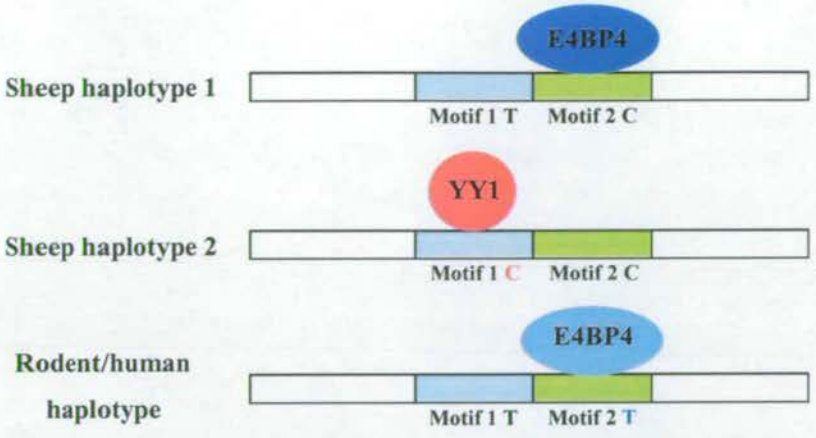


Figure 7.1 *Potential interactions of YY1 and E4BP4 on the PrP promoter*

Motif 1T = Suffolk sheep, rodent, bovine & human PrP promoter motif 1 sequence (TCATTTT), Motif 1C = Cheviot sheep polymorphic motif 1 sequence (CCATTTT), Motif 2C = bovine & ovine motif 2 sequence (TTACGTAA), Motif 2T = rodent & human motif 2 sequence (TTATGTAA). E4BP4 = the bZIP repressor protein (or E4BP4 transcription factor) also known as nuclear factor of interleukin 3 (NF-IL3). YY1 = the Yin Yang 1 transcription factor.

The binding of YY1 to the Cheviot promoter in preference to E4BP4 was supported by DNase I footprinting experiments carried out during this study which indicated that the area of the Cheviot promoter containing the variant motif 1 may have been protected by the binding of a nuclear protein. In contrast, the DNA sequence containing motif 2 did not appear to be occupied, a result consistent with YY1 binding to the Cheviot promoter at the variant motif 1, preventing the potential binding of E4BP4 to motif 2 (Sheep haplotype 2, Figure 7.1). However, as the DNase I footprints of the Cheviot promoter were only carried out on three occasions, further repeats are necessary and could also be investigated using the Suffolk promoter which contains the wild type motif 1.

It is unclear how the binding of the E4BP4 and YY1 transcription factors would affect PrP gene expression, or disease susceptibility. The polymorphic change which results in the binding of YY1, appears not to be linked to any obvious scrapie susceptible or resistant genotypes (Dr Gerry O'Neill, NPU, personal

communication). In addition, the deletion of motifs 1 and 2 in a variety of mammalian PrP promoters has failed to reveal any significant effect on reporter gene expression (Baybutt & Manson, 1997, Funke-Kaiser *et al.*, 2001, Inoue *et al.*, 1997, Mahal *et al.*, 2001, O'Neill *et al.*, 2003, Saeki *et al.*, 1996). However, these deletion experiments would not have taken into account potential interactions between factors bound to the motifs and other transcription factors, which could play an important role in their influence on PrP gene expression. A number of techniques are available to further analyse potential interactions between two proteins including the yeast two hybrid system, co-immunoprecipitation, the chromatin immunoprecipitation (ChIP) assay and the TranSignal™ TF-TF Interaction Array II. These techniques could be used to characterise any potential interaction between YY1 and E4BP4, and may provide a clearer picture of the role that these two factors play in the control of PrP gene expression.

This study has used a selection of *in vitro* techniques to investigate the binding of transcription factors to selected motifs within the ovine PrP promoter. These techniques enable the identification of proteins that bind to free radiolabeled oligonucleotides, however, they may not accurately replicate the *in vivo* situation. Eukaryotic DNA is condensed into a highly ordered complex (a nucleosome) with small basic proteins termed histones (Travers, 1994). This complex is known as chromatin and serves to package and organise chromosomal DNA within the nucleus (Travers, 1994, Wade, Pruss & Wolffe, 1997). The positioning of nucleosomes along eukaryotic DNA can determine the accessibility of specific DNA sequences to transcription factors, thus providing an additional level of gene transcription regulation (Kornberg & Lorch, 1992, Kornberg & Lorch, 1999, Workman & Buchman, 1993, Workman & Kingston, 1998). Therefore, the *in vitro* assays used in this study may not be representative of the whole picture as they do not accurately reflect the role of DNA packaging on gene transcription.

The positioning of nucleosomes can be regulated by specific enzymes termed histone acetyltransferases (HATs) that acetylate histone proteins in a reversible reaction, causing the DNA within the nucleosome to become less condensed (Cosma *et al.*, 1999, Kornberg & Lorch, 1999). This acetylation event may act to promote the binding of other transcription factors or it may enable the basal transcription complex

machinery to bind to the promoter. Interestingly, all of the mammalian PrP gene promoters with the exception of the human and rat promoters contain a potential binding motif for the transcription co-activator protein p300 (GGGAGTG), which has been shown to function as a HAT (Korzus *et al.*, 1998, Ogryzko *et al.*, 1996).

7.2 Concluding remarks

This thesis has analysed the non-coding regions of the PrP gene with a view to expanding current knowledge of the role that these elements play in the regulation of PrP gene expression. The main aims of this thesis were addressed by the following questions:

1. Is endogenous PrP^c expressed in the ovine cell lines?
2. What is the role of alternative polyadenylation of ovine PrP mRNA in control of PrP^c expression?
3. Do sequence specific transcription factors bind to sequence motifs within the ovine PrP promoter?
4. Do single nucleotide polymorphisms within the ovine PrP promoter affect transcription factor binding?
5. Is the binding of transcription factors to the ovine PrP promoter cell/ tissue specific?

All of these aims, with the exception of the analysis of the role of alternative polyadenylation in PrP gene expression, were successfully fulfilled during this study. At the time of writing this was the first study to have analysed the sequence of the ovine PrP promoter in depth, with a view to identifying potential transcription factor binding motifs. Therefore, the results presented in this thesis constitute the first demonstration of sequence specific binding of transcription factors to the ovine PrP promoter. It is widely accepted that eukaryotic gene expression is primarily regulated at the transcriptional level by the binding of general transcription factors (GTFs) to TATA and/or Initiator elements. The binding of GTFs to promoters and the rate of

transcription are further regulated by the binding of other transcription factors acting as either activators or repressors of transcription.

Work carried out during this thesis has identified putative Initiator and downstream promoter elements which are conserved in all of the mammalian PrP promoters analysed, thus elucidating a potential mechanism by which PrP gene expression may be initiated. Specific binding to the ovine PrP promoter by transcription factors known to interact with the basal transcription complex was also demonstrated. The binding of factors such as SP-1 and AP-2 may represent one way in which the rate of transcription of the PrP gene is further regulated. In addition, for the first time single nucleotide polymorphisms within the ovine PrP promoter were shown to be biologically significant, resulting in the differential binding of transcription factors to the ovine PrP gene promoter.

Binding to the four promoter motifs identified by Westaway *et al.* (1994a) was analysed and specific binding to motifs 1 and 2, by the transcription factors YY1 and E4BP4 respectively, was demonstrated. In addition, a hypothetical model for the binding of YY1 and E4BP4 to the ovine promoter was produced, indicating a mechanism by which the expression of the PrP gene could be further regulated. Furthermore, the binding of YY1 and E4BP4 to the ovine PrP gene promoter could influence the rate of gene transcription either by interacting with the basal transcription complex or by interactions with other transcription factors, for example, SP-1 or AP-2. Further studies are therefore required to analyse the role that these factors play in the regulation of PrP gene expression.

The influence of transcription factors like YY1 and E4BP4 on the expression of PrP^{c} could be subtle, and tissue-specific differences in where PrP^{sc} infectivity is found have been documented and seem to be related to PrP genotype. For example, Houston, Halliday, Jeffrey *et al.* (2002) showed clear genotype-specific differences in the distribution and relative levels of PrP^{sc} accumulation between New Zealand sheep experimentally challenged with SSBP/1 scrapie. Potentially these differences in the distribution of PrP^{sc} could be caused by variations in the level of PrP^{c} expression in sheep of different scrapie genotypes and this could have serious implications for any mechanism by which scrapie can spread through the body. For example, using the conformational conversion model (Section 1.2.1), if PrP^{c} was in

plentiful supply infectivity could potentially spread faster than if PrP^c was limited (Jarrett & Lansbury, 1993). However, few studies have analysed the tissue distribution and relative level of PrP^c in sheep. The analysis of PrP^{sc} and PrP^c in neuronal tissues has become routine, however, methods for the analysis of PrP^c in non-neuronal tissues, where the relative levels are frequently much lower than those observed in the brain, are much less well defined. Horiuchi *et al.* (1995) showed that PrP^c was detectable in non-neuronal tissues such as spleen, lymph node, lung, kidney and intestine albeit at much lower levels than observed with neuronal tissue. Furthermore, Moudjou, Frobert, Grassi *et al.* (2001) analysed the distribution of PrP^c in a variety of non-neuronal tissues of sheep of different scrapie genotypes. However, the number of animals used was very low and as such it was not possible to determine whether there was a relationship between genotype and the tissue specific expression of PrP^c (Moudjou *et al.*, 2001). Therefore, studies into the distribution and relative levels of PrP^c in a variety of peripheral tissues of sheep of different breeds and scrapie genotypes are required to further our understanding of the role of PrP^c expression in the pathogenesis of scrapie.

Finally, during this study no differences in protein-DNA binding were observed with nuclear extracts prepared from the cell cultures derived from sheep of known natural scrapie susceptible (sA80BR) and resistant (pA80BR) genotypes. However, any genotype specific differences in PrP^c expression could be manifested in a tissue-specific manner that was not analysed with the cell cultures tested in this study. Therefore, it may be prudent to repeat the gel shift assays carried out in this study with nuclear extracts derived from other tissues and in particular from tissue-types known to be involved in the spread of infectivity, such as follicular dendritic cells and Langerhans cells. In addition, the binding of YY1 and E4BP4 to motifs 1 and 2, respectively, could be analysed in gel shift assays with nuclear extracts derived from frozen archive tissues from scrapie pathogenesis studies. These studies may highlight potential differential genotype related gel shift results, which could help to explain the distribution of PrP^{sc}.

7.3 Future projects

This thesis has identified potential sequence elements within the ovine PrP gene promoter that may act to initiate and regulate gene transcription, and has demonstrated sequence specific binding to a number of these elements. Therefore, the work carried out in this thesis has expanded upon the existing knowledge of the role of the PrP promoter in the regulation of gene expression and therefore contributes to our understanding of how disease development might be controlled in sheep. It has not provided data to associate the molecular interaction of nuclear proteins with the control of PrP gene expression.

One obvious question arising from this thesis is therefore concerning the functional significance of the T / C polymorphism in motif 1 for healthy or scrapie-affected sheep. To begin answering this important question it would be advisable to show differential transcription rates due to the polymorphism, for which one could employ two alternative methods of analysis. The first method would be based on the haplotype-specific chromatin immunoprecipitation (HaploChIP) technique (Knight, Keating, Rockett *et al.*, 2003) which uses the detection of RNA polymerase II loading on the promoter to indicate the level of transcriptional activity. In short, experiments could be conducted with ovine cell lines or tissue samples known to be heterozygous for the motif 1 T/C polymorphism, in which the DNA and nuclear proteins would be cross-linked using formaldehyde, sonicated and the resulting DNA fragments would be immunoprecipitated with an antibody directed against RNA polymerase II. The DNA fragments purified from immunoprecipitation would then be selectively quantified using real time PCR analysis with primers specific for either the wild type (T) or polymorphic (C) motif 1 sequence. In this way the loading of RNA polymerase II and therefore the level of gene transcription could be determined for the wild type and variant sequences of motif 1. This approach would represent the *in vivo* environment of the chromatin within the normal cell. The second method would be to use *in vitro* transcription containing ovine PrP promoter plasmid constructs with either the wild type (T) or variant (C) sequences of motif 1 carried out in the presence of nuclear extracts derived from ovine cell cultures or ovine tissue samples. This experiment would allow the level of gene transcription to be

determined for the wild type and variant motif 1 sequences in an *in vitro* environment.

In addition, to the more long term studies detailed above a number of shorter term experiments could be conducted to answer the following questions that have arisen from the work carried out during this thesis:

1. What are the specific requirements for the formation of the transcription initiation complex on the PrP promoter?
2. Are these requirements maintained or altered with regard to the observed polymorphisms in the ovine and murine PrP promoter?
3. Is there a species and/or tissue specificity in these protein-DNA interactions, which could help to establish a PrP-specific association between early transcriptional regulation and levels of PrP mRNA?
4. Are the complexes formed between the motifs and the transcription factors responsive to environmental changes, i.e. cytokine activation of cells?

These questions could be addressed by the following studies:

1. Analysis of the Inr & DPEs elements identified in the ovine and murine promoters:

Binding to the PrP promoter Inr and DPEs could be analysed using gel shift assays. Any bound transcription factors could be identified using gel super-shift assays with antibodies specific for basal transcription complex proteins, most of which are commercially available (at least for the mouse & human proteins). Potential interactions between any newly characterised factors could be analysed, and furthermore interactions between these factors and AP-2, SP-1 and YY1 / E4BP4 could also be assessed.

2. Investigation into the effect of species-specific polymorphic changes within AP-2 clusters on transcription factor binding:

The murine PrP promoter also contains an AP-2 motif cluster and breed specific polymorphisms within this cluster alter the number of available AP-2

binding motifs (Baybutt & Manson, 1997). Potential differential binding to the polymorphic variants of this cluster could be analysed. These experiments could help to elucidate any effects that these polymorphisms may have on murine PrP gene expression.

3. Analysis of tissue-specific binding of transcription factors to binding motifs within the ovine PrP gene promoter:

Differential binding to the ruminant motif 2 was revealed using ovine liver and brain-derived NEs. Similar differences were also observed between murine brain and liver tissue NEs. Therefore, it may be that different factors are bound to this motif in different tissue-types, as E4BP4 has been shown to compete with other bZIP transcription factors for the same binding motif. Gel super-shift assays with the anti-E4BP4 antibody with NEs produced from these different tissue-types could reveal the differential binding of bZIP transcription factors to motif 2 in different tissues. In addition, the analysis of binding to motif 2 with NEs produced from other cell types, in particular from peripheral tissues shown to be important in scrapie disease control, such as follicular dendritic cells could provide further insights into the tissue-specific regulation of PrP. Furthermore, in order to assess any differential genotype related affects which could explain the distribution of PrP^{sc}, binding to motif 2 could also be assessed in nuclear extracts prepared from stored tissues from TSE pathogenesis studies.

4. Investigation into the role of pro-inflammatory cytokines on the binding of STAT transcription factors to the PrP promoter:

A conserved binding motif for the STAT family of transcription factors was identified in all of the mammalian PrP promoters and a polymorphic change of C to A within this motif in sheep is linked to the positive line of NPU Cheviot sheep. Gel shift assays carried out to date indicated only weak binding to the variant form of this motif, however STAT proteins are inducible transcription factors which are activated to translocate to the nucleus only in response to extracellular stimuli such as proinflammatory cytokines. Shift assays could be performed with NEs prepared from murine and ovine cells which have been pre-treated with IFN-γ, which has been

shown to induce the translocation of STAT transcription factors to the nucleus (Darnell, 1997, Levy & Darnell, 2002). These experiments could provide further insights into the role of the STAT motif in PrP gene expression and could highlight a possible function for these factors in disease susceptibility.

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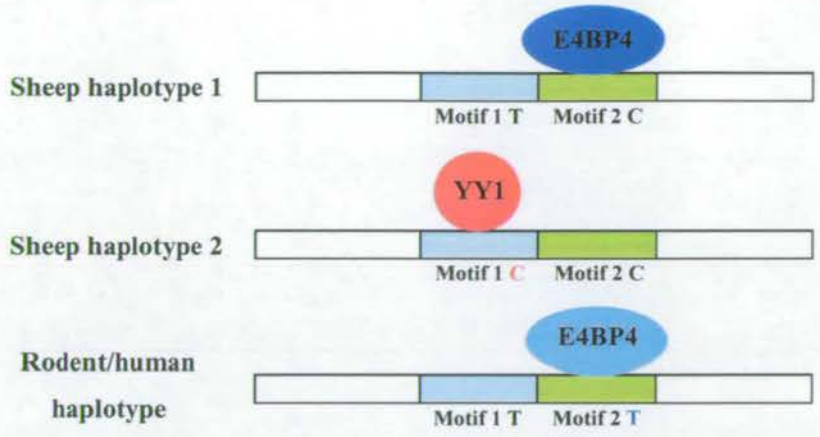

Figure 7.1 *Potential interactions of YY1 and E4BP4 on the PrP promoter*

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3. Do sequence specific transcription factors bind to sequence motifs within the ovine PrP promoter?
4. Do single nucleotide polymorphisms within the ovine PrP promoter affect transcription factor binding?
5. Is the binding of transcription factors to the ovine PrP promoter cell/ tissue specific?

All of these aims, with the exception of the analysis of the role of alternative polyadenylation in PrP gene expression, were successfully fulfilled during this study. At the time of writing this was the first study to have analysed the sequence of the ovine PrP promoter in depth, with a view to identifying potential transcription factor binding motifs. Therefore, the results presented in this thesis constitute the first demonstration of sequence specific binding of transcription factors to the ovine PrP promoter. It is widely accepted that eukaryotic gene expression is primarily regulated at the transcriptional level by the binding of general transcription factors (GTFs) to TATA and/or Initiator elements. The binding of GTFs to promoters and the rate of

transcription are further regulated by the binding of other transcription factors acting as either activators or repressors of transcription.

Work carried out during this thesis has identified putative Initiator and downstream promoter elements which are conserved in all of the mammalian PrP promoters analysed, thus elucidating a potential mechanism by which PrP gene expression may be initiated. Specific binding to the ovine PrP promoter by transcription factors known to interact with the basal transcription complex was also demonstrated. The binding of factors such as SP-1 and AP-2 may represent one way in which the rate of transcription of the PrP gene is further regulated. In addition, for the first time single nucleotide polymorphisms within the ovine PrP promoter were shown to be biologically significant, resulting in the differential binding of transcription factors to the ovine PrP gene promoter.

Binding to the four promoter motifs identified by Westaway *et al.* (1994a) was analysed and specific binding to motifs 1 and 2, by the transcription factors YY1 and E4BP4 respectively, was demonstrated. In addition, a hypothetical model for the binding of YY1 and E4BP4 to the ovine promoter was produced, indicating a mechanism by which the expression of the PrP gene could be further regulated. Furthermore, the binding of YY1 and E4BP4 to the ovine PrP gene promoter could influence the rate of gene transcription either by interacting with the basal transcription complex or by interactions with other transcription factors, for example, SP-1 or AP-2. Further studies are therefore required to analyse the role that these factors play in the regulation of PrP gene expression.

The influence of transcription factors like YY1 and E4BP4 on the expression of PrP^{c} could be subtle, and tissue-specific differences in where PrP^{sc} infectivity is found have been documented and seem to be related to PrP genotype. For example, Houston, Halliday, Jeffrey *et al.* (2002) showed clear genotype-specific differences in the distribution and relative levels of PrP^{sc} accumulation between New Zealand sheep experimentally challenged with SSBP/1 scrapie. Potentially these differences in the distribution of PrP^{sc} could be caused by variations in the level of PrP^{c} expression in sheep of different scrapie genotypes and this could have serious implications for any mechanism by which scrapie can spread through the body. For example, using the conformational conversion model (Section 1.2.1), if PrP^{c} was in

plentiful supply infectivity could potentially spread faster than if PrP^c was limited (Jarrett & Lansbury, 1993). However, few studies have analysed the tissue distribution and relative level of PrP^c in sheep. The analysis of PrP^{sc} and PrP^c in neuronal tissues has become routine, however, methods for the analysis of PrP^c in non-neuronal tissues, where the relative levels are frequently much lower than those observed in the brain, are much less well defined. Horiuchi *et al.* (1995) showed that PrP^c was detectable in non-neuronal tissues such as spleen, lymph node, lung, kidney and intestine albeit at much lower levels than observed with neuronal tissue. Furthermore, Moudjou, Frobert, Grassi *et al.* (2001) analysed the distribution of PrP^c in a variety of non-neuronal tissues of sheep of different scrapie genotypes. However, the number of animals used was very low and as such it was not possible to determine whether there was a relationship between genotype and the tissue specific expression of PrP^c (Moudjou *et al.*, 2001). Therefore, studies into the distribution and relative levels of PrP^c in a variety of peripheral tissues of sheep of different breeds and scrapie genotypes are required to further our understanding of the role of PrP^c expression in the pathogenesis of scrapie.

Finally, during this study no differences in protein-DNA binding were observed with nuclear extracts prepared from the cell cultures derived from sheep of known natural scrapie susceptible (sA80BR) and resistant (pA80BR) genotypes. However, any genotype specific differences in PrP^c expression could be manifested in a tissue-specific manner that was not analysed with the cell cultures tested in this study. Therefore, it may be prudent to repeat the gel shift assays carried out in this study with nuclear extracts derived from other tissues and in particular from tissue-types known to be involved in the spread of infectivity, such as follicular dendritic cells and Langerhans cells. In addition, the binding of YY1 and E4BP4 to motifs 1 and 2, respectively, could be analysed in gel shift assays with nuclear extracts derived from frozen archive tissues from scrapie pathogenesis studies. These studies may highlight potential differential genotype related gel shift results, which could help to explain the distribution of PrP^{sc}.

7.3 Future projects

This thesis has identified potential sequence elements within the ovine PrP gene promoter that may act to initiate and regulate gene transcription, and has demonstrated sequence specific binding to a number of these elements. Therefore, the work carried out in this thesis has expanded upon the existing knowledge of the role of the PrP promoter in the regulation of gene expression and therefore contributes to our understanding of how disease development might be controlled in sheep. It has not provided data to associate the molecular interaction of nuclear proteins with the control of PrP gene expression.

One obvious question arising from this thesis is therefore concerning the functional significance of the T / C polymorphism in motif 1 for healthy or scrapie-affected sheep. To begin answering this important question it would be advisable to show differential transcription rates due to the polymorphism, for which one could employ two alternative methods of analysis. The first method would be based on the haplotype-specific chromatin immunoprecipitation (HaploChIP) technique (Knight, Keating, Rockett *et al.*, 2003) which uses the detection of RNA polymerase II loading on the promoter to indicate the level of transcriptional activity. In short, experiments could be conducted with ovine cell lines or tissue samples known to be heterozygous for the motif 1 T/C polymorphism, in which the DNA and nuclear proteins would be cross-linked using formaldehyde, sonicated and the resulting DNA fragments would be immunoprecipitated with an antibody directed against RNA polymerase II. The DNA fragments purified from immunoprecipitation would then be selectively quantified using real time PCR analysis with primers specific for either the wild type (T) or polymorphic (C) motif 1 sequence. In this way the loading of RNA polymerase II and therefore the level of gene transcription could be determined for the wild type and variant sequences of motif 1. This approach would represent the *in vivo* environment of the chromatin within the normal cell. The second method would be to use *in vitro* transcription containing ovine PrP promoter plasmid constructs with either the wild type (T) or variant (C) sequences of motif 1 carried out in the presence of nuclear extracts derived from ovine cell cultures or ovine tissue samples. This experiment would allow the level of gene transcription to be

determined for the wild type and variant motif 1 sequences in an *in vitro* environment.

In addition, to the more long term studies detailed above a number of shorter term experiments could be conducted to answer the following questions that have arisen from the work carried out during this thesis:

1. What are the specific requirements for the formation of the transcription initiation complex on the PrP promoter?
2. Are these requirements maintained or altered with regard to the observed polymorphisms in the ovine and murine PrP promoter?
3. Is there a species and/or tissue specificity in these protein-DNA interactions, which could help to establish a PrP-specific association between early transcriptional regulation and levels of PrP mRNA?
4. Are the complexes formed between the motifs and the transcription factors responsive to environmental changes, i.e. cytokine activation of cells?

These questions could be addressed by the following studies:

1. Analysis of the Inr & DPEs elements identified in the ovine and murine promoters:

Binding to the PrP promoter Inr and DPEs could be analysed using gel shift assays. Any bound transcription factors could be identified using gel super-shift assays with antibodies specific for basal transcription complex proteins, most of which are commercially available (at least for the mouse & human proteins). Potential interactions between any newly characterised factors could be analysed, and furthermore interactions between these factors and AP-2, SP-1 and YY1 / E4BP4 could also be assessed.

2. Investigation into the effect of species-specific polymorphic changes within AP-2 clusters on transcription factor binding:

The murine PrP promoter also contains an AP-2 motif cluster and breed specific polymorphisms within this cluster alter the number of available AP-2

binding motifs (Baybutt & Manson, 1997). Potential differential binding to the polymorphic variants of this cluster could be analysed. These experiments could help to elucidate any effects that these polymorphisms may have on murine PrP gene expression.

3. Analysis of tissue-specific binding of transcription factors to binding motifs within the ovine PrP gene promoter:

Differential binding to the ruminant motif 2 was revealed using ovine liver and brain-derived NEs. Similar differences were also observed between murine brain and liver tissue NEs. Therefore, it may be that different factors are bound to this motif in different tissue-types, as E4BP4 has been shown to compete with other bZIP transcription factors for the same binding motif. Gel super-shift assays with the anti-E4BP4 antibody with NEs produced from these different tissue-types could reveal the differential binding of bZIP transcription factors to motif 2 in different tissues. In addition, the analysis of binding to motif 2 with NEs produced from other cell types, in particular from peripheral tissues shown to be important in scrapie disease control, such as follicular dendritic cells could provide further insights into the tissue-specific regulation of PrP. Furthermore, in order to assess any differential genotype related affects which could explain the distribution of PrP^{sc}, binding to motif 2 could also be assessed in nuclear extracts prepared from stored tissues from TSE pathogenesis studies.

4. Investigation into the role of pro-inflammatory cytokines on the binding of STAT transcription factors to the PrP promoter:

A conserved binding motif for the STAT family of transcription factors was identified in all of the mammalian PrP promoters and a polymorphic change of C to A within this motif in sheep is linked to the positive line of NPU Cheviot sheep. Gel shift assays carried out to date indicated only weak binding to the variant form of this motif, however STAT proteins are inducible transcription factors which are activated to translocate to the nucleus only in response to extracellular stimuli such as proinflammatory cytokines. Shift assays could be performed with NEs prepared from murine and ovine cells which have been pre-treated with IFN-γ, which has been

shown to induce the translocation of STAT transcription factors to the nucleus (Darnell, 1997, Levy & Darnell, 2002). These experiments could provide further insights into the role of the STAT motif in PrP gene expression and could highlight a possible function for these factors in disease susceptibility.

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APPENDIX 1

General solutions

Agar plates

32 g of LB agar (Roche, UK) to 1L of dH_2O, split to 3 x 500 ml Duran bottles (~330 ml)
Autoclaved for 15 minutes at 121°C
Melted in microwave at low setting for 20 minutes with occasional shaking
Cooled to 55°C and poured, allowed to cool on bench and stored at + 4 °C

Ampicillin

0.5 g ampicillin powder (Sigma, UK) to 10 ml dH_2O
Aliquoted into 500 µl lots in 1.5 ml microcentrifuge tubes and stored at – 20°C

Ampicillin plates

When agar cooled to 55°C, ampicillin (Sigma, UK) was added to a final concentration of
1 µl / ml. The agar was then mixed and poured as above

Ampicillin X-Gal/ isopropyl-beta-D-thiogalactopyranoside (IPTG) plates for blue-white selection

When agar cooled to 55°C ampicillin (Sigma, UK) was added as above
IPTG (Roche, UK) was added to a final concentration of 0.5 mM and X-Gal (Roche, UK) was added to a final concentration of 80 µg/ml
The agar was then mixed and poured as above

Luria-Bertani (LB) broth

20 g of LB broth base (Roche, UK) to 1L of dH_2O
Autoclaved for 15 minutes at 121°C, added ampicillin (Sigma, UK) if required at concentration of 1 µl / ml

1 % Agarose gels

One gram of agarose (Roche, UK) dissolved in 100 ml 1x TBE buffer
Heated in microwave on medium high setting for 1.5 minutes
Ethidium bromide (Sigma, UK) was added to a final concentration of 0.5 µg / ml
Gel was allowed to set at room temperature for 1-2 hours
Ran in Multipurpose Gel Electrophoresis Tank (IBI, UK)

4 % acrylamide sequencing gel

17.5 g of urea (BDH, UK)
4 ml 10x TBE buffer
5.7 ml of 40 % acrylamide solution (BDH, UK)
700 µl 10 % APS solution (BDH, UK)
15 ml of dH_2O
Mixed until dissolved and added 63 µl TEMED (Sigma, UK) whilst stirring

6 % acrylamide gel for DNase I footprinting assay
48 g of urea (BDH, UK)
15 ml 40 % acrylamide/bisacrylamide solution, 37.5:1 (2.6 % C) (Bio-Rad, USA)
10 ml 10x TBE buffer
37 ml dH_2O
700 µl 10 % APS solution (BDH, UK)
Mixed until dissolved and added 50 µl TEMED (Sigma-Aldrich, UK) whilst stirring

4 % DNA retardation gel for gel shift and super-shift assays
32.4 ml dH_2O
2.5 ml 40 % acrylamide/bisacrylamide solution, 37.5:1 (2.6 % C) (Bio-Rad, USA)
1.5 ml 40 % acrylamide solution (Bio-Rad, USA)
2 ml 10x TBE buffer
1.25 ml 80 % Glycerol solution (BDH, UK)
300 µl 10 % APS solution (BDH, UK)
Mixed until dissolved and added 20 µl TEMED (Sigma, UK) whilst stirring

Miniprep solution A
50 mM glucose (BDH, UK)
25 mM tris-HCl (pH 8)
10 mM EDTA (pH 8.5)
Prepared in 100 ml aliquots without glucose, autoclaved, added glucose and stored at 4°C

Miniprep solution B
7 ml dH_2O
2 ml 1M NaOH
1 ml 10 % SDS
Prepared fresh for each preparation

Miniprep solution C
100 ml 5M potassium acetate
19.1 ml glacial acetic acid (BDH, UK)
47 ml dH_2O
Autoclaved at 121°C for 15 minutes and stored at room temperature

10% Ammonium persulphate (APS)
0.5 g APS (BDH, UK)
Added to 5 ml dH_2O and stored at 4°C covered in foil

10 % sodium dodecyl sulphate (SDS)
20 g SDS powder (BDH, UK)
Added to 180 ml dH_2O
Made up to 200 ml with dH_2O when completely dissolved

Gel loading buffer 5x
30 g Ficoll 400 (BDH, UK)
1 ml 10 % SDS
8 µl 0.5 M EDTA (pH 8)
0.25 g Orange G (Sigma, UK)

50X TAE buffer
242 g tris base (Roche, UK)
57.1 ml glacial acetic acid (BDH, UK)
100 ml 0.5 M EDTA (pH 8)
Made up to 1L with dH_2O

10x TBE buffer
108 g tris base (Roche, UK)
55 g boric acid (BDH, UK)
40 ml 0.5 M EDTA (pH 8)
Made up to 1L with dH_2O

Ethidium bromide
One 10 mg ethidium bromide tablet (Sigma, UK) into 1 ml dH_2O and made up to 2 ml with dH_2O

TE buffer, pH 7.4
10 mM tris-HCl, pH 7.4
0.25 mM EDTA (pH 8)

1M Tris buffer
12.1 g tris base (Roche, UK) into 80 ml distilled dH_2O
Adjusted pH to 7.5 with concentrated HCl and made up to 100 ml with dH_2O

1M NaCl
5.84 g NaCl (BDH, UK) into 80 ml distilled dH_2O
Dissolved and made up to 100 ml with dH_2O

0.5 M EDTA (pH 8)
186.1 g EDTA.$2H_2O$ (BDH, UK)
Made up to 800 ml with dH_2O
Added solid NaOH (BDH, UK) and stir well to pH 7.8
Added 10 % NaOH solution to pH 8
Made up to 1L with dH_2O and autoclaved for 15 minutes at 121°C

3M NaAc (pH 5.2)
408.1 g NaAc.$3H_2O$ (BDH, UK) into 800 ml dH_2O
Adjusted pH to 5.2 with glacial acetic acid
Made up to 1L with dH_2O
Dispensed into 100 ml aliquots and autoclaved at 121°C for 15 minutes

3M NaAc (pH 7)

408.1 g NaAc.$3H_2O$ (BDH, UK) into 800 ml dH_2O
Adjusted pH to 7 with dilute acetic acid
Made up to 1L with dH_2O, dispensed into 100 ml aliquots and autoclaved at 121°C for 15 minutes

RNase A Solution

One µl RNase A solution (Roche, UK) was diluted in 100 µl of dH_2O and stored at –20 °C until use

Complete mini – Protease Inhibitor solution

One tablet (Roche, UK) dissolved in 1 ml dH_2O and mixed by vortexing. Stored at 4 °C.

Solutions for tissue culture

Fetal calf serum (FCS)

500 ml heat inactivated (56°C for 45 minutes) FCS (Globepharm, UK) aliquoted into 50 ml lots, stored at -20°C. Centrifuged immediately before use at 2,000 rpm for 10 minutes

Standard complete medium (SCM)

500 ml Dulbecco's MEM + Glutamax (Invitrogen, UK)
50 ml FCS (Globepharm, UK)
Penicillin / Streptomycin to final concentration of 100 units / ml i.e. stock 100 units per
0.01 ml therefore added 5 ml Pen / Strep to 500 ml medium

Nerve growth factor (NGF) complete medium (NCM)

500 ml Dulbecco's MEM + Glutamax (Invitrogen, UK)
50 ml FCS (Globepharm, UK)
Penicillin / Streptomycin (Invitrogen, UK) to final concentration of 100 units / ml
Nerve growth factor to final concentration of 10 ng/ml, therefore 100 µg stock taken up to 10 ml in dH_2O and 0.5 ml NGF (Sigma, UK) added to 500 ml culture medium to give a final concentration of 10 ng/ml

Freezing medium

30 ml Standard Culture Medium (SCM)
1.5 ml FCS (Globepharm, UK)
5 ml DMSO (Sigma, UK)
Aliquoted into 8 x 6 ml aliquots and stored at – 20°C

Trypsin/ versene (TVP)

4 ml 25 % trypsin (Invitrogen, UK)
16 ml 0.02 % versene (Invitrogen, UK)

0.1 % Gelatin
0.4 g gelatin (Biorad, UK) into 400 ml dH_2O
Autoclaved for 15 minutes at 121°C and stored at 4°C

Bio-Rad protein assay II dye reagent
20 ml Bio-Rad protein assay II dye reagent
80 ml dH_2O
Mixed and filtered through Whatman No. 1 filter paper, stored in glass bottle at room temperature for maximum of two weeks.

Bio-Rad protein assay protein standard
Lyophilised bovine serum albumin reconstituted in 20 ml dH_2O (1.4 mg/ml)
Aliquoted into 20 x 1 ml in 0.5 ml microcentrifuge tubes and stored at –20 °C

Solutions for Immunoprecipitations

Wash Buffer 1
2.4 ml 1M tris-HCl (pH 7.5)
7.2 ml 1M NaCl
0.48 ml Nonidet P-40 (Sigma, UK)
38 ml dH_2O

Wash Buffer 2
2.4 ml 1M tris-HCl (pH 7.5)
24 ml 1M NaCl
0.48 ml Nonidet P-40 (Sigma, UK)
21.1 ml dH_2O

Wash Buffer 3
0.24 ml 1M tris-HCl (pH 7.5)
23.8 ml dH_2O
All wash buffers were prepared fresh for each precipitation

Solutions for SDS-polyacrylamide-gel electrophoresis (SDS-PAGE)

NuPage MES SDS running buffer (20x)
50 ml running buffer (Invitrogen, UK)
950 ml dH_2O
500 µl NuPage Antioxidant (Invitrogen, UK)

NuPage LDS Sample buffer (4x)
200 µl sample buffer (Invitrogen, UK)
800 µl dH_2O

Antioxidant
NuPage Antioxidant (Invitrogen, UK)

Sample reducing agent
NuPage Sample reducing agent (10x) (Invitrogen, UK)

Protein standards
Pre-stained protein molecular weight standards (Invitrogen, UK)
14,300-200,000 molecular weight range, lyophilized powder
Added 500 µl dH_2O and flash frozen in 5 µl aliquots, stored at – 20°C

SDS-PAGE Gels
NuPage Pre-cast 4-12 % Bis-Tris gel (Invitrogen, UK)

Solutions for chemiluminescence blotting

NuPage Transfer buffer (20x)
25 ml NuPage transfer buffer (Invitrogen, UK)
424.5 ml dH_2O
50 ml methanol (BDH, UK)
0.5 ml NuPage antioxidant (Invitrogen, UK)

TBS
6.05 g tris base (Roche, UK)
8.76 g NaCl (BDH, UK)
Added dH_2O to 800 ml
Adjusted pH to 7.5 with HCl and made up to 1L with dH_2O

TBST
For one blot: 0.25 ml Tween 20 (Roche, UK) in 250 ml TBS

1 % Blocking solution
For one blot: 1 ml blocking agent (Roche, UK) in 10 ml TBS

0.5 % Blocking solution
For one blot: 5 ml blocking agent (Roche, UK) in 100 ml TBS

Primary Antibody
6H4 anti-PrP monoclonal antibody (Prionics, CH). Lyophilized powder, added 1 ml dH_2O and flash frozen in 15 µl aliquots, stored at – 70°C

Secondary Antibody
Peroxidase conjugated affinipure rabbit anti-mouse IgG (Jackson Immuno-research Lab Inc, Stratech, UK). Lyophilized powder, added 500 µl dH_2O and flash frozen in 5 µl aliquots, stored at – 20°C

Detection solution
BM Chemiluminescence blotting substrate (POD) (Roche, UK)
Pre-warmed Solution A (Roche, UK) for 30 minutes at 25°C.

APPENDIX 2

PrP promoter transcriptional initiator (Inr) and downstream promoter elements (DPEs)

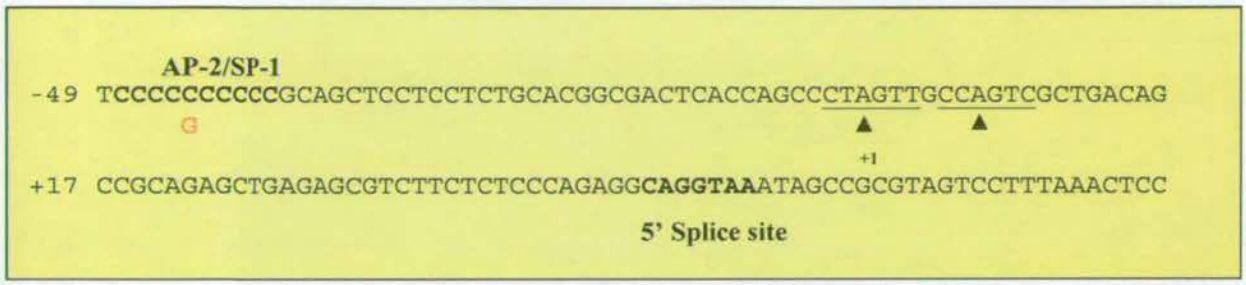

Figure A1 *Ovine PrP gene Inr elements and potential transcriptional start sites*

Polymorphic changes are indicated by a base change (different colour) underneath the wild type sequence (Suffolk sheep, Genbank = U67922). Arrowheads indicate location of transcription start sites as experimentally shown by Westaway *et al.* (1994a). Suffolk sheep PrP gene promoter sequence from -49 to +81 bp, numbering based on the use of the 5' transcription start site (Genbank = U67922). Also highlighted is the polymorphic AP-2/SP-1 site seen in Cheviot sheep (O'Neill *et al.*, 2003). Underlined sequences indicate the location of potential initiator (Inr) elements. 5' splice site is indicated in bold text.

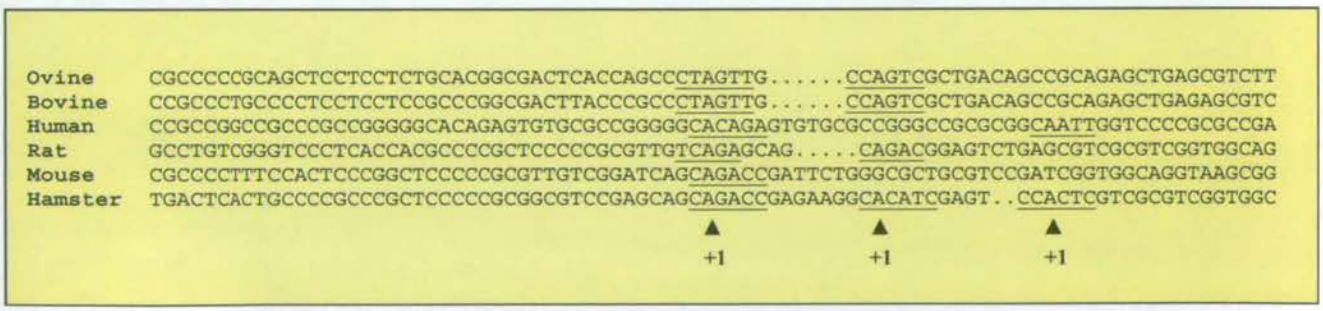

Figure A2 *Mammalian PrP gene Inr elements and potential transcriptional start sites*

Arrowheads indicate location of transcription start sites as experimentally shown by Westaway *et al.* (1994a) (ovine), (Inoue *et al.*, 1997) (bovine), (Baybutt & Manson, 1997) (murine), (Funke-Kaiser *et al.*, 2001) (human). Underlined sequences indicate the location of potential initiator (Inr) elements. NB: Artificial gaps were introduced into the above sequences in order to line-up potential transcription start sites using the GCG (Wisconsin) using local pile-up program.

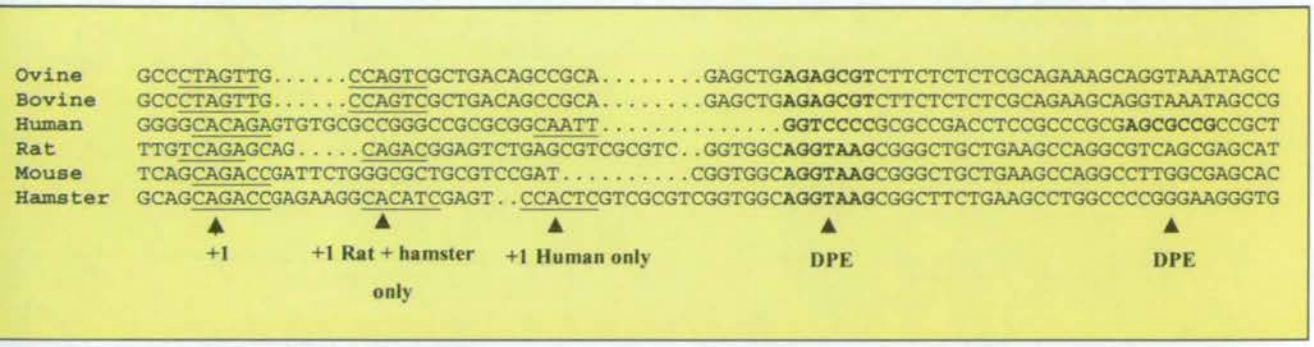

Figure A3 *Line-up of mammalian PrP gene Inr & DPEs and potential transcriptional start sites*

Arrowheads indicate location of transcription start sites as experimentally shown by Westaway *et al.* (1994a) (ovine), (Inoue *et al.*, 1997) (bovine), (Baybutt & Manson, 1997) (murine), (Funke-Kaiser *et al.*, 2001) (human). Underlined sequences indicate the location of potential initiator (Inr) elements. Bold sequences indicate the location of putative downstream promoter elements (DPEs). NB: Artificial gaps were introduced into the above sequences in order to line-up potential transcription start sites using the GCG (Wisconsin) using local pile-up program.